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CORRECTIONS.

In the tables on pages 22, 23, and 24, Vol. lx, No. 1, May, 1924, for P read H_2PO_4 .

ON WALDEN INVERSION.

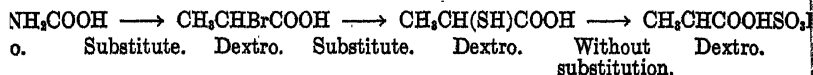
II. THE OPTICAL ROTATION OF THIOLACTIC AND CORRESPONDING α -SULFOPROPIONIC ACIDS.

By P. A. LEVENE AND L. A. MIKESKA.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 3, 1924.)

The aim of the work of which the present report represents a part was discussed in a previous communication.¹ It was there reported that when *l*-2-mercapto-octane was oxidized to 2-octane sulfonic acid, the latter rotated in an opposite direction from that of the parent substance. It was also suggested that in other substances in which the radicals attached to the asymmetric carbon atom were of a different polarity, the oxidation of the thio derivative into the sulfonic acid derivative might remain without influence on the direction of rotation. A case of that nature is here reported. *d*-Thiolactic acid was oxidized to α -sulfopropionic acid which rotated in the same direction as the parent substance.



Comparing the result of the reactions observed on methylhexyl carbinol and on lactic acid it was noticed that the conversion of the bromide into the thio derivative was accompanied with a change of direction of rotation in the alcohol and in the acid. Also, the oxidation of the thio derivative into the sulfonic acid was accompanied with a change of direction of rotation in the case of the alcohol and not in that of the acid. Further work on this problem is in progress.

¹ Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1924, lix, 473.

EXPERIMENTAL.

α -Thiolactic Acid.—The *l*-bromopropionic acid used in this preparation was prepared by the action of nitrosyl bromide on α -alanine according to the directions of Fischer and Warburg.² The acid had a rotation of $[\alpha]_D^{20} = -21.65^\circ$. This acid (25 gm.) was dissolved in 65 cc. of water and neutralized with 8.58 gm. of sodium carbonate. The mixture was then treated with 28.6 gm. of potassium xanthate and allowed to stand at room temperature overnight. 25 cc. of concentrated HCl were then added and the solution was heated on a steam bath for half an hour to decompose any unchanged xanthic acid. The solution was extracted with ether, and dried over sodium sulfate. On removal of the ether, a crystalline residue remained. The total yield of the xanthate was 30 gm. The optical rotation in ether solution was:

$$[\alpha]_D^{20} = +\frac{1.82^\circ \times 100}{1 \times 4.73} = +38.5^\circ$$

The xanthate was converted into thiolactic acid, according to Einar Büllmann.³ The xanthic acid was dissolved in a mixture of 200 cc. of absolute alcohol and 75 cc. of concentrated aqueous ammonia and allowed to stand at room temperature for 48 hours. The alcohol and ammonia were then distilled off under reduced pressure, the residue was rendered alkaline with ammonia and extracted with ether to remove the xanthogenamide. The residue was then acidified with hydrochloric acid and extracted with ether. The extract was dried over Na_2SO_4 , the ether removed, and the residue fractionated. The fraction, boiling up to 95° , ($p = 16$ mm.), was discarded. The second fraction distilled at 95 – 100° ($p = 16$ mm.) and had a specific rotation of $[\alpha]_D^{20} = +19.90^\circ$ without solvent. In another experiment the bromosuccinic acid had a rotation of $[\alpha]_D^{20} = +33.81^\circ$, and the thiolactic acid prepared from it had a rotation of $[\alpha]_D^{20} = +38.32^\circ$.

d - α -Sulfopropionic Acid.—11 gm. of *d*- α -thiolactic acid ($[\alpha]_D^{20} = +20^\circ$) were dissolved in 100 cc. of water and cooled to 0° . Bromine was then added, little by little, with cooling until no more bromine was absorbed. The total volume of the solution at

² Fischer, E., and Warburg, O., *Ann. Chem.*, 1905, cccxi, 171.

³ Büllmann, E., *Ann. Chem.*, 1905, cccxxxix, 371.

the end of the reaction was 110 cc. The rotation of the solution was determined in a 1 dm. tube and was found to be $+1.10^\circ$. The solution was concentrated to a small volume under diminished pressure, treated with an excess of barium carbonate, and filtered hot. On cooling, a white crystalline precipitate appeared. On concentration two more crops of crystals were obtained. The third fraction was recrystallized from water, dried, and analyzed.

0.1050 gm. substance: 0.0128 gm. H_2O and 0.0434 gm. CO_2 .

0.2966 " " 0.2318 " $BaSO_4$ (for S).

0.0989 " " 0.0792 " " (" Ba).

$C_3H_4O_3BaS$. Calculated. C 12.43, H 1.39, Ba 47.48, S 11.07.

Found. " 11.18, " 1.35, " 47.12, " 10.73.

0.5914 gm. of the above salt, equivalent to 0.314 gm. of *d*- α -sulfopropionic acid, was dissolved in 10 per cent hydrochloric acid, diluted to 10 cc., and gave a rotation of

$$[\alpha]_D^{20} = \frac{+0.33^\circ \times 100}{1 \times 3.14} = +10.51^\circ$$

GROWTH AND REPRODUCTION UPON SIMPLIFIED FOOD SUPPLY.

IV. IMPROVEMENT IN NUTRITION RESULTING FROM AN INCREASED PROPORTION OF MILK IN THE DIET.*

By H. C. SHERMAN AND H. L. CAMPBELL.

(From the Department of Chemistry, Columbia University, New York.)

(Received for publication, March 20, 1924.)

The work of Osborne and Mendel, Hart and Steenbock, and McCollum and coworkers has made familiar the fact that wheat does not constitute an adequate diet because of its insufficient content of calcium and fat-soluble vitamin, and that these deficiencies are readily supplied by milk so that adequate nutrition is possible on a food supply consisting of these two foods alone.

Previous papers from this laboratory^{1,2,3} have shown that when dry milk constitutes one-sixth and ground whole wheat five-sixths of the food mixture the diet (Diet A) is adequate for growth and reproduction in the rat, normal nutrition having been maintained in some of our rat families for eight generations on this diet. If, however, the proportion of milk be increased from one-sixth to one-third of the solids of the wheat-and-milk mixture (Diet B), better nutrition results.

That distinct improvements in nutrition can be brought about by alterations in a diet which is already adequate seems to us to be a fact of sufficient significance to warrant careful study. To be able to demonstrate conclusively, and to measure in quantitative terms, improvements in nutrition over what is already adequate may facilitate the advancement of our knowledge of

* Published as Contribution No. 443 from the Department of Chemistry, Columbia University.

¹ Sherman, H. C., Rouse, M. E., Allen, B., and Woods, E., *J. Biol. Chem.*, 1921, xlii, 503.

² Sherman, H. C., and Muhlfeld, M., *J. Biol. Chem.*, 1922, liii, 41.

³ Sherman, H. C., and Crocker, J., *J. Biol. Chem.*, 1922, liii, 49.

nutrition in more than one direction. For the fuller understanding of the relations of nutrition to physical efficiency it seems important to know as much as possible about the difference between adequate and optimum nutrition; and it is by quantitative work in this region that we may best hope to advance our knowledge of such difficult questions as, for instance, the significance of vitamin C in the nutrition of those animals which seem to be adequately nourished on food lacking this factor but to thrive somewhat better when it is supplied. By the same general methods we may also study quantitatively the requirements of optimum adult nutrition as contrasted with the requirements of growth or the minimum requirements of maintenance.

We have, therefore, continued the investigation of the nutritive effects of the two diets above mentioned (Diet A, adequate, and Diet B, better), and have measured quantitatively on fairly large numbers of individuals certain differences in growth, in successful reproduction, and other evidences of efficiency of nutrition resulting from the feeding of the two diets, respectively, for several successive generations to families of rats from the same stock and which in all other respects lived under identical conditions and were treated in exactly the same way. Two independent series of such comparisons have been made, both of which are still being continued, but have now been carried sufficiently far to justify the presentation of the data and the drawing of the conclusions which follow.

EXPERIMENTAL DATA AND DISCUSSION.

The experimental animals were white rats, bred in this laboratory from Osborne and Mendel stock. As stated in their papers, this stock was originally obtained from the rat colony of the Wistar Institute, and during several generations of breeding in the laboratories of Osborne and Mendel has been rendered somewhat more resistant to disease by the systematic rejection of any weaklings appearing in the breeding stock. On the other hand there has been no attempt nor desire to increase the average size by selection of large individuals for breeding, and our records show average results in close accord with the weight curves published by Donaldson and King in 1915.⁴

⁴ Donaldson, H. H., *The rat*, Philadelphia, 1915, 69.

In the first series the comparison began with rats taken at random from the same stock and was continued by keeping sufficient of their descendents to give from ten to twenty females on each diet in each generation with enough males to make satisfactory groups for breeding, the number of males being usually less than the number of females. The numbers just stated refer to rats kept upon their respective diets until after the end of their reproductive lives; growth and development were studied upon considerably larger numbers.

Although the random choice of animals with which this comparison began was carefully kept free from any conscious selection, later study of the records of these and other groups suggested a possibility that the families assigned to Diet A may have been naturally endowed with slightly smaller size and somewhat greater breeding capacity than those assigned to Diet B.

In the second series any such possibility was guarded against by starting the experiment with parallel lots each of one male and three females, all the eight animals being of one litter and so divided into two lots that these had the same average initial weight. As all the original animals of the second series thus had exactly the same family history, and as they were of very even size, it is believed that differences appearing in the second series can with confidence be attributed to the difference in food alone.

That this is essentially true of the first series also is strongly indicated by the fact that the same differences between the two diets are found in both series of experiments.

The two diets used in the experiments described in this paper were:

Diet A (Laboratory No. 16).—A mixture of one-sixth whole milk powder with five-sixths ground whole wheat and sodium chloride to the extent of 2 per cent of the weight of the wheat.

Diet B (Laboratory No. 13).—A mixture of one-third whole milk powder with two-thirds ground whole wheat and sodium chloride to the extent of 2 per cent of the weight of the wheat.

Other Experimental Conditions.—Only distilled water was used. The animals were kept in all-metal cages. No bedding was used except for mothers with young less than 2 weeks old, and then consisted only of pure cellulose. The young were

separated from the mothers at the uniform "weaning" age of 4 weeks and each rat was weighed at this time and at weekly intervals thereafter. Food consumption was determined for the same weekly intervals.

The points of quantitative comparison chiefly considered in this paper are: The rate and economy of growth during the 4 weeks following weaning, the general comparison of average size at different ages from weaning time to middle age, the time required to reach sexual maturity as indicated by the ages of females at birth of their first young, the duration of reproductive life, the success in bearing and rearing young, and the growth of the young during the suckling period.

TABLE I.

Rate of Growth as Shown by Gain in Weight during the 28 Days Following Weaning—the 5th to 8th Weeks, Inclusive, of the Life of the Rat.

Description.	No. of cases on each diet.	Diet A.	Diet B.	Differences.
Males.				
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
1st series.....	100	35.0 \pm 0.7	82.0 \pm 0.8	47.0 \pm 1.0
2nd "	100	53.7 \pm 1.5	84.0 \pm 1.5	30.3 \pm 2.1
Combined.....	200	44.3 \pm 0.9	82.9 \pm 0.8	38.6 \pm 1.2
Females.				
1st series.....	100	30.0 \pm 0.6	59.0 \pm 0.6	29.0 \pm 0.8
2nd "	100	44.0 \pm 1.3	65.8 \pm 0.9	21.8 \pm 1.6
Combined.....	200	37.0 \pm 0.8	62.4 \pm 0.6	25.4 \pm 0.9

1. *Rate of Growth.*—It will be seen from Table I that for each sex and for each series the growth on Diet B was decidedly more rapid than on Diet A, and that the difference is so many times greater than its probable error as to make the demonstration indubitable.

Since growth is thus plainly more rapid on Diet B, the question next arises whether this more rapid growth calls for an arithmetically proportional increase in the food intake or whether Diet B induces a more economical as well as a more rapid growth.

2. *Efficiency of Growth.*—The data of both series of experiments (Table II) lead to the conclusion that Diet B is more efficiently

utilized for growth in that it induces a larger gain in weight per 1,000 calories of food consumed; and the difference, being in both series well over ten times its probable error, is large enough to constitute an unquestionable demonstration.

No distinction of sex is here made in connection with the data bearing on this point because our previous work³ has shown it to be unnecessary, and because in the experiments here considered the young rats were caged in litters containing both sexes in order that breeding might occur as soon as sexual maturity was attained.

3. *Average Size.*—Both the diets here studied permitted growth to normal adult size. Diet B, however, resulted in slightly larger average size at all ages both during growth and after growth had been completed (Table III). It is interesting to

TABLE II.

Efficiency of Growth as Shown by Grams of Gain in Body Weight per 1,000 Calories of Food Consumed during the 5th to 8th Weeks, Inclusive, of the Rats' Lives.

Description.	No. of lots on each diet.	Diet A.	Diet B.	Differences.
		gm.	gm.	gm.
1st series.....	50	54.8 \pm 0.9	74.6 \pm 0.8	19.8 \pm 1.2
2nd "	50	66.2 \pm 0.9	79.4 \pm 0.9	13.2 \pm 1.2
Combined.....	100	60.5 \pm 0.7	77.0 \pm 0.6	16.5 \pm 1.0

note that the average weights on Diet A are slightly below, and on Diet B slightly above the Donaldson-King averages of 1915.⁴ The differences in size induced by the two diets here studied, while consistent and doubtless significant, are probably not of such great significance as are the differences in vigor reflected by the breeding records (Table IV).

4. *Time Required to Reach Maturity.*—The time required to reach maturity as measured by the average ages of the females on the two diets at the birth of their first young, is shown for the females of the first four generations of the first series in the first part of Table IV. Because of the special interest attaching to the comparison of these data with those for the same individuals on the other points discussed below, the comparison in Table IV is confined to the four groups of females on each

TABLE III.

Average Weights of Rats on the Two Diets, Compared at Various Ages.

Age.	Males.		Females.	
	Diet A.	Diet B.	Diet A.	Diet B.
<i>days</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
28	36.4	42.6	34.8	40.5
30	38.6	47.0	36.6	44.3
40	53.1	74.2	49.0	66.5
50	70.7	105.2	63.6	89.3
60	91.1	138.7	77.6	110.4
70	110.9	169.2	91.7	130.2
80	130.2	192.2	104.4	147.4
90	147.3	210.4	115.8	162.8
100	162.4	224.3	126.6	168.8
110	175.6	231.3	135.5	175.4
120	187.1	247.1	146.0	182.1
130	197.8	255.9	153.7	186.1
140	207.5	264.6	160.9	189.9
150	215.3	270.3	165.1	194.5
180	237.3	288.2	178.2	203.9
210	250.1	300.3	187.9	210.6
250	259.7	306.3	196.2	216.9
300	268.6	314.9	201.6	222.2
350	270.4	315.1	204.6	223.7
400	269.6	311.4	202.9	228.6

TABLE IV.

Average Breeding Records of 64 Females on Diet A and 53 on Diet B. (Representing the First Four Generations on Each Diet—Series I).

	Diet A.	Diet B.	Differences.
Age at birth of first young, <i>days</i> ...	162 \pm 3	116 \pm 3	46 \pm 4.6
Duration of reproductive life, <i>days</i>	204 \pm 9	324 \pm 14	120 \pm 16
Average No. of young reared.....	5.6 \pm 0.6	16.1 \pm 1.1	10.5 \pm 1.2
Average weight of young at weaning, <i>gm.</i>	32.9 \pm 0.6	41.1 \pm 0.4	8.2 \pm 0.7

diet whose breeding records were known to be entirely completed at the time of writing. Separate consideration of the first, second, third, and fourth generations of the series shows no significant deviations from the relation shown by the general averages as

presented in Table IV. It is plain that Diet B induced greater vigor as shown by earlier maturity.

This conclusion is confirmed by the data from larger numbers of individuals which are collected in Table V. Both Tables IV and V show differences which are so many times larger than their probable errors as to give us entire confidence that a true difference of nutritive condition induced by the larger proportion of milk in Diet B than in Diet A has here been demonstrated to exist and to be capable of quantitative investigation.

TABLE V.

Average Breeding Records of Females of Both Series Combined So Far As Completed at Time of Writing.

	Diet A.		Diet B.		Differences.
	No. of cases.	Average.	No. of cases.	Average.	
Age at birth of first young, <i>days</i>	200	155 \pm 1.5	200	112 \pm 1.1	43 \pm 1.9
Duration of reproductive life, <i>days</i> .	115	186 \pm 7.7	129	322 \pm 8.9	136 \pm 11.7
Average No. of young reared.....	115	5.8 \pm 0.5	129	18.1 \pm 0.8	12.3 \pm 0.9
Average weight of young at weaning,* <i>gm</i>	673	34.0 \pm 0.5	2,337	40.3 \pm 0.3	6.3 \pm 0.6
Males†, <i>gm</i>	231	33.8 \pm 0.2	1,226	40.3 \pm 0.2	6.5 \pm 0.3
Females†, <i>gm</i>	290	32.6 \pm 0.1	1,298	39.2 \pm 0.2	6.6 \pm 0.2

* The data on this line are for the young of the same females whose records are given on the two preceding lines.

† These data for weight of young at the uniform "weaning" age of 4 weeks are averages of all cases of such young on these two diets in our colony during the year Dec. 12, 1922, to Dec. 11, 1923, inclusive, an exact year being included in the comparison in order that any possible seasonal influence should apply equally to the families on the two diets.

5. *Duration of Reproductive Life.*—As has been pointed out by McCollum, diet may influence the onset of senility. In our experiments we have not attempted to determine the date of onset of senility from the appearance of the animal, but have recorded the age at which each female lost the power of breeding as inferred from the date of birth of her last young, the females being always remated as soon as each litter of young was weaned.

We have considered the time from the beginning of the first pregnancy to the end of the last lactation as the duration of reproductive life. In counting this from the dates of birth of the first and last litters, 21 days is allowed for pregnancy preceding the birth of the first litter, and 21 days for the suckling of the last litter if any of them are reared. (As already noted we allow the young to remain with the mother for 4 weeks; but during the 4th week suckling is rarely seen.)

On Diet A the reproductive life of the females of the series shown in Table IV ceased on the average at 345 days; on Diet B, not until the age of 419 days. Thus the improvement in nutrition induced by Diet B extended the prime of life in both directions, postponing old age in the same individuals in which it had induced earlier maturity, and very materially prolonging the duration of reproductive life. This has proved equally true in both series (Tables IV and V).

6. Success in Reproduction and the Rearing of Young.—The success is here expressed by showing the average number of total young per female which the females on the two diets reared to weaning age. This is shown for the most directly comparable groups whose records are yet complete in Table IV, and for the larger groups (including these and others) in Table V.

Although individual variability is here a large factor, the examination of the data makes it entirely plain that the improved nutrition resulting from Diet B very greatly increased the capacity of the mothers to produce and rear young.

The females on Diet B not only reared a larger number of young but also a larger percentage of the young born to them.

On both diets some females failed to rear any young, but such cases of total failure were much fewer on Diet B than on Diet A. On Diet A, 48 per cent failed to rear young; on Diet B, 19 per cent.

7. Average Weights of Young at Weaning.—The weights of the young at birth do not vary greatly and in most of our work the individual weighings have been begun when the young are 4 weeks old. At this age the diet is found to have exerted a considerable influence upon the growth of the young during the suckling period (Tables IV and V). It is noteworthy that the females on Diet B have at the same time suckled a much larger

number of young and brought them to a larger average size at weaning time.

Numbers of Cases Studied.—Each of the data discussed in this paper is an average of observations on from 53 to 1,298 individuals.

TABLE VI.

Food Consumption of Rats in Terms of Calories per Gram of Rat per Day, at Different Ages.

Age period.	Diet A.		Diet B.	
	Series I. 1st-4th generations.	General average.	Series I. 1st-4th generations.	General average.
<i>wks.</i>				
5- 6	0.46	0.47	0.49	0.49
7- 8	0.42	0.42	0.40	0.41
9- 10	0.36	0.37	0.33	0.35
11- 12	0.33	0.33	0.29	0.30
13- 14	0.30	0.30	0.26	0.27
15- 16	0.29	0.28	0.24	0.25
17- 18	0.28	0.27	0.24	0.24
19- 20	0.27	0.26	0.22	0.24
21- 22	0.25	0.25	0.22	0.23
23- 24	0.26	0.25	0.21	0.23
25- 26	0.26	0.24	0.22	0.23
27- 30	0.25	0.24	0.22	0.22
31- 34	0.23	0.23	0.21	0.22
35- 38	0.23	0.23	0.22	0.22
39- 42	0.22	0.22	0.21	0.23
43- 46	0.21	0.22	0.22	0.22
47- 50	0.21	0.22	0.21	0.22
51- 54	0.21	0.22	0.21	0.21
55- 58	0.22	0.22	0.20	0.21
59- 62	0.22	0.23	0.20	0.21
63- 66	0.21	0.22	0.21	0.21
67- 70	0.20	0.21	0.21	0.21
71- 74	0.22	0.21	0.20	0.20
75- 78	0.23	0.24	0.21	0.21

Food Consumption.—That the greatly improved breeding records shown on Diet B over those on Diet A cannot be attributed primarily to a merely greater food consumption is shown by the records briefly summarized in Table VI from which it will be seen that the food consumption per unit of body weight

was practically the same on the two diets—once the period of rapid growth was passed—*viz.*, approximately 0.22 calorie of total food per gram of body weight per day, not including in the average the latter part of pregnancy and the period of lactation. Systematic discussion of the relation of food consumption to the life cycle is deferred until a later communication, the present purpose being merely to point out that Diet B induced improvement in nutrition in other ways than merely through inducing a larger consumption of food.

Cumulative Breeding Record.—As explained above, the initial animals of Series II were all from one litter, divided into two equal groups of one male and three females each. Starting thus exactly simultaneously and with precisely the same family history, each group and all its descendents were kept on its respective diet and allowed to multiply without restriction at such rates as the nutritive conditions resulting from their respective diets would permit, until 1 year from the date of birth of the original animals. The result was that at this time those on Diet A had a total of 77 descendents, and those on Diet B had 361 descendents, an increase of 368 per cent in the cumulative breeding record on Diet B over that on Diet A.

Such use of the cumulative breeding record to compare the nutritive values of two diets both of which are adequate is well adapted to accentuate differences which might otherwise remain obscure, since the differences both in rate of reaching maturity and in successful reproduction are thus made to work together for the intensification of the numerical contrast in the final result. This method, however, makes severe demands upon time, space, and control of all experimental conditions; and if attempted upon a smaller scale the individual variability which is so striking a feature of breeding records might often lead to erroneous conclusions.

Growth and reproduction records such as those summarized in Tables I to IV are, therefore, recommended as more generally applicable.

In these summaries of our data of such records we have accompanied the quantitative statements of findings with estimates of their probable errors as computed by the classical method. Just how precise this method may be as applied to observa-

tions of this character cannot be stated with entire certainty at the present time, because elaborate studies of the frequency distribution of large numbers of such data have not yet been made; but for the kinds of measurements of which we have largest numbers, namely, weights of young at weaning time and rates of growth during the following 4 week period, the frequency distribution approximates so closely the theoretical (the symmetrical bell-shaped distribution curve) as to lend confidence to the use of the probable error as an aid to judgment in the interpretation of the results. In view of this, the fact that all the differences here discussed have been measured for many individuals and found to be many times their probable errors, adds much to the confidence with which we may summarize the findings.

SUMMARY.

Starting with a diet which was shown to be adequate in that it supported growth, reproduction, and successful suckling of the young for generation after generation, it was found that an increase in the proportion of milk in this already adequate diet resulted in the following evidences of improved nutrition.

1. More rapid growth, particularly as measured by the gain in weight during the period following weaning.

2. More efficient growth during the same period as shown by greater gain in weight per 1,000 calories of food consumed.

3. Somewhat larger average size at all ages.

4. Earlier maturity as shown by age of female at birth of her first young.

5. Longer duration of reproductive life, old age being deferred in the same individuals in which earlier maturity has been induced.

6. Greater success in the rearing of young as shown by increase both in numbers and in percentage of young reared, and by decrease in percentage of females failing to bear and rear young.

7. Better growth of young during the suckling period as shown by larger average weight at the uniform weaning age adopted.

The better of the two diets here considered is probably capable of further improvement.

Evidently there is not only a line to be drawn but a wide zone

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CONCERNING THE MECHANISM OF ACIDOSIS IN ANESTHESIA.

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Not long ago (1) data were presented which showed that subsequent to the period of ether anesthesia in dogs there was an increased elimination of bases (sodium and potassium). The increase was especially marked when morphine was given prior to the administration of the ether. As a consequence of the general result it became a matter of interest to know what acid, if any, had accompanied the increment of bases. Several experiments similar to those already published were, therefore, carried out, and the urine was examined with this end in view.

Lactic acid, acetone bodies, sulfuric acid, and phosphoric acid were sought for in the first two experiments. Lactic acid and acetone bodies were not detected and sulfuric acid showed no significant variations. Phosphoric acid, on the other hand, showed pronounced variations and attention was, therefore, concentrated upon it. Short (2) has already shown that a causal relation between acetone bodies and a low plasma bicarbonate is unlikely and Leake, Leake, and Koehler (3) have reported that there are no significant changes in the concentration of the acetone bodies of the blood as a result of ether anesthesia. It was regarded as superfluous to examine the blood for changes in the alkali reserve and hydrogen ion concentration since the variations which occur are now well established (1, 4, 5, 6). Fast-ing dogs were employed which were catheterized as the periods required.

The data (Table I) indicate a close parallelism between the excretion of bases and that of phosphoric acid in the anesthetic and postanesthetic periods, and there can be little doubt that the same relation would have been observed in the previously

reported experiments had the urine been examined for phosphoric acid. Chloroform appears to exert a more decided effect on

TABLE I.

Experiment No.	Period.		Condition.	Na ₂ SO ₄ + K ₂ SO ₄	H ₂ PO ₄
	No.	Length.			
		<i>hrs.</i>		<i>gm.</i>	<i>gm.</i>
1	1	24	Normal.		0.531
	2	24	"	0.646	0.408
	3a	3	Ether.	0.067	0.023
	3a	Calculated to 24 hr. basis.		0.536	0.187
	3b	21	Normal.	1.286	0.713
	3a+3b			1.353	0.736
	4		Normal.		0.452
2	1	24	"		
	2	24	"	0.824	0.708
	3a	3	Ether preceded 45 min. by 10 mg. morphine per kg.	0.559	0.357
	3a	Calculated to 24 hr. basis.		3.63	2.32
	3b	21	Normal.	1.00	0.777
	3a+3b			1.56	1.13
3	1	24	Normal.		0.472
	2	24	"	1.22	0.723
	3a	3	Chloroform.	0.268	0.202
	3a	Calculated to 24 hr. basis.		2.15	1.62
4	1	24	Normal.		
	2	24	"	1.25	0.777
	3a	3	Chloroform.	0.121	0.079
	3a	Calculated to 24 hr. basis.		0.871	0.569
	3b	21	Normal.	1.89	1.24
	3a+3b			2.01	1.32

Na, K, and P excretion than ether, but we have not followed this point further.

In order to obtain more precise information concerning the phosphoric acid metabolism during anesthesia, experiments upon dogs with bladder fistulas were carried out and the total phosphorus excreted was determined in the periods as indicated in the table.¹ Food was withheld for 20 hours before each experiment.

The micro method of Pregl, somewhat modified, was employed. The modification consisted in the process of ashing. This was done by evaporating 1 cc. of urine in a Pyrex tube over a free flame with 5 drops of sulfuric acid and about 1 cc. of 30 per cent hydrogen peroxide (Merck's "superoxol").

In the case of simple ether anesthesia, anuria (Experiments 7 and 12) or varying degrees of oliguria develop. Consequently, most of the simple ether experiments show a diminished phosphorus excretion during anesthesia. There are exceptions to this rule. For example, in the two experiments with Dog 4 there was an increase in the rate of phosphorus excretion. It would appear, then, that the increased phosphorus excretion on the experimental day in the long experiments first cited must be due to an increased rate of excretion occurring some time subsequent to the cessation of the anesthetic. This period cannot be immediately after the anesthesia period because in those experiments in which the urine of postanesthetic periods was examined the rate of phosphorus excretion in these periods was lower than the control rate.

When morphine was given as a preliminary to ether, in eight experiments out of ten there was a diminished phosphorus excretion in the morphine period followed by an increased excretion in the ether period. This subsequent increase was most marked in several of the experiments with Dog 2. Dog 1 in two instances responded rather with an increased chloride excretion during the ether period than with an increased phosphorus excretion. An attempt will be made to explain this augmented excretion of phosphorus in discussing the fat experiments which follow.

The source of the phosphoric acid noted was investigated next. We expected to find a decrease in the phosphorus content

¹ Urea and chlorides were determined in all the experiments here described and will be published elsewhere because of their bearing on the theories of urine excretion.

of one or more tissues, and selected as those most likely to show changes, the brain, the liver, and the muscles.

TABLE II.

Experiment No.	Dog No.	Time of collection.	Urine per min.	P per min.	Remarks.
Ether experiments.					
7	1	<i>p.m.</i>	<i>cc.</i>	<i>mg.</i>	Ether 2.05-3.16 p.m.
		1.05-2.05	0.068	0.014	
		2.05-3.16	0.000		
		4.40-5.30	0.076	0.006	
10	1	12.50-1.40	0.121	0.079	Ether 1.40-3.15 p.m.
		1.55-3.15	0.020	0.0033	
12	1	1.55-2.45	1.11	0.065	Ether 2.47-3.55 p.m.
		2.55-3.55	0.00		
		4.47-5.27	0.09	0.0083	
		5.53-6.23	1.03	0.0068	
		200 cc. water by stomach tube at 12.30 p.m.			
13	4	12.20-1.10	0.132	0.342	Ether 1.10-2.25 p.m.
		1.15-2.25	0.077	0.493	
		4.34-5.24	0.176	0.378	
		8.16-8.57	0.127	0.126	
14	3	2.18-2.28	0.166	0.152	Ether 3.08-5.02 p.m.
		3.22-4.22	0.066	0.029	
		4.22-5.02	0.105	0.118	
15	4	1.21-2.11	0.83	0.479	Ether 2.11-3.20 p.m.
		2.20-3.20	0.085	0.542	
		3.20-4.00	0.112	0.467	
		4.38-5.18	0.139	0.018	
125 cc. water by stomach tube at 12.30 p.m.					
17	3	1.01-1.41	0.59	0.393	Ether 1.41-2.50 p.m.
		2.00-2.50	0.13	0.268	
		4.22-4.52	0.21	0.356	
19	2	1.04-1.44	0.168	0.216	Ether 1.44-2.54 p.m.
		1.54-2.54	0.018	0.021	
		3.53-4.23	0.210	0.016	
		5.16-5.46	0.400	0.106	

TABLE II—*Concluded.*

Experiment No.	Dog No.	Time of collection.	Urine per min.	P per min.	Remarks.
Morphine-ether experiments.					
4	2	<i>p.m.</i> 2.40-3.10	<i>cc.</i> 0.17	<i>mg.</i> 0.085	10 mg. morphine sulfate per kg. at 3.11 p.m.
		3.21-4.01	0.19	0.054	
6	2	1.31-2.11	0.099	0.034	10 mg. morphine sulfate per kg. at 2.21 p.m.
		2.21-3.21	0.081	0.012	
		3.21-4.31	0.049	0.085	Ether 3.21-5.41 p.m.
		4.31-5.41	0.064	0.327	
9	2	1.45-2.45	0.101	0.061	10 mg. morphine sulfate per kg. at 2.45 p.m.
		3.05-4.05	0.043	0.031	
		4.15-5.15	0.150	0.297	Ether 4.05-5.15 p.m.
11	2	1.35-2.05	2.4	0.0348	10 mg. morphine sulfate per kg. at 2.05 p.m.
		2.27-3.07	0.26	0.0914	
		3.15-4.45	0.24	0.673	Ether 3.09-4.45 p.m.
		400 cc. water by stomach tube at 12.15 p.m.			
16	2	1.53-2.33	0.92	0.0284	10 mg. morphine sulfate per kg. at 2.34 p.m.
		2.34-3.44	0.07	0.0087	
		3.49-4.59	0.15	0.0456	Ether 3.44-4.59 p.m.
		4.59-5.09	0.19	0.0433	
		150 cc. water by stomach tube at 1.35 p.m.			
18	4	2.33-3.13	0.102	0.164	10 mg. morphine sulfate per kg. at 3.35 p.m.
		3.42-4.42	0.045	0.025	
		4.47-5.47	0.154	0.568	Ether 4.42-5.47 p.m.
20	2	1.39-2.09	2.1	0.294	10 mg. morphine sulfate per kg. at 2.10 p.m.
		2.25-3.20	0.0	0.000	
		3.23-3.48	0.136	0.175	Ether 3.56-5.54 p.m.
		4.07-5.22	0.05		
		5.22-5.54	0.24	0.467	80 cc. water at 10.00 and 11.00 a.m., and 12.00, 1.00, and 2.15 p.m.
21	4	1.30-2.10	0.22	0.355	10 mg. morphine sulfate per kg. at 2.25 p.m.
		2.32-3.32	0.11	0.143	
		3.38-3.48	0.17	0.478	Ether 3.32-4.38 p.m.

White rats were anesthetized under bell jars by passing in a mixture of air and ether vapor. At the end of 2 hours they were killed by concentrating the ether. Controls were killed by strangulation. The bodies were allowed to rest for a time in order to diminish the bleeding which occurs when the liver is removed. In all but the last experiment the respective tissues of the control and etherized animals were assembled in single samples for the analyses. In the last experiment the muscle and liver of each rat were analyzed individually. This was done in order to obtain some information concerning individual variations in presumably uniform animals. All samples were ashed with a mixture of sulfuric and nitric acids and aliquot portions were taken for the analyses. For determining phosphorus the molybdic acid-magnesia mixture method as described by Treadwell (7) in which the phosphorus is finally weighed as the pyrophosphate was employed. For the ash determinations a portion of the digest was evaporated and then ignited below red heat. In those cases where sodium and potassium were determined the method was that outlined in the paper mentioned (1).

Regarded in connection with the Meyer-Overton theory of narcosis it would appear that the nervous system lipoids might be involved. A perusal of the data of the following experiments, however, indicates that ether anesthesia is without influence upon the phosphorus content of the brain. The variations observed in the phosphorus contents of the liver and muscles were surprising. Instead of a decrease in the case of one or both there was an increase in the case of the former and a decrease in the case of the latter. The results are given in per cent.

Experiment 1.—Animals of unknown history and averaging 163 gm. in weight, purchased in one lot, were used. Six females (non-pregnant) were employed for the controls and six males for determining the action of the ether.

Controls.					Ether.				
Muscle.		Liver.		Brain.	Muscle.		Liver.		Brain.
P	$\frac{\text{Na}_2\text{SO}_4}{\text{K}_2\text{SO}_4}$	P	$\frac{\text{Na}_2\text{SO}_4}{\text{K}_2\text{SO}_4}$	P	P	$\frac{\text{Na}_2\text{SO}_4}{\text{K}_2\text{SO}_4}$	P	$\frac{\text{Na}_2\text{SO}_4}{\text{K}_2\text{SO}_4}$	P
0.768	1.22	0.738	1.14	1.09	0.712	1.21	0.826	1.15	1.07

Experiment 2.—Animals of unknown history were employed. Four females (non-pregnant) averaging 150 gm. in weight served as controls and six males averaging 160 gm. served for determining the action of the ether.

Controls.					Ether.				
Muscle.		Liver.		Brain.	Muscle.		Liver.		Brain.
P	Na_2SO_4 + K_2SO_4	P	Na_2SO_4 + K_2SO_4	P	P	Na_2SO_4 + K_2SO_4	P	Na_2SO_4 + K_2SO_4	P
0.804	1.07	0.722	0.96	1.08	0.736	1.12	0.777	0.96	1.08

Experiment 3.—Animals of unknown history and averaging 250 gm. in weight were employed. Four males served as controls and four females (non-pregnant) for determining the action of the ether.

Controls.					Ether.				
Muscle.		Liver.		Brain.	Muscle.		Liver.		Brain.
P	Na_2SO_4 + K_2SO_4	P	Na_2SO_4 + K_2SO_4	P	P	Na_2SO_4 + K_2SO_4	P	Na_2SO_4 + K_2SO_4	P
0.750	1.07	0.694	0.958	1.03	0.729	1.13	0.737	0.965	1.01

Experiment 4.—The animals here employed were non-pregnant females averaging 250 gm. in weight and of unknown age. They were purchased from E. V. B. Douredoure, Philadelphia. Fifteen served as controls and twenty-four for determining the action of ether.

Controls.					Ether.				
Muscle.		Liver.		Brain.	Muscle.		Liver.		Brain.
P	Ash.	P	Ash.	P	P	Ash.	P	Ash.	P
0.691	1.45	0.747	1.40	1.02	0.676	1.44	0.758	1.43	1.01

Experiment 5.—The animals herein employed were similar to those of Experiment 4, but they were males and averaged about 275 gm. in weight.

Controls.				Ether.				
Rat No.	Muscle P.	Liver P.	Liver Ash.	Rat No.	Time.	Muscle P.	Liver P.	Ash.
					min.			
270	0.685	0.670	1.48	289	20	0.736	0.669	1.29
272	0.721	0.751	1.46	296	40	0.686	0.719	1.39
284	0.728	0.733	1.45	265	75	0.733	0.788	1.47
285	0.704	0.698	1.44	262	90	0.665	0.818	1.44
286	0.746	0.604	1.31	264	95	0.701	0.826	1.45
287	0.739	0.648	1.28	298	95	0.720	0.708	
				297	120	0.701	0.734	1.43
				295	120	0.699	0.755	1.39
				288	120	0.694	0.766	1.44
				268	120	0.698	0.827	1.46
				263	125	0.690	0.864	1.42
Average....	0.721	0.684	1.40			0.702	0.770	1.42

In Experiments 1, 2, and 3 the animals which served for the controls and those to which ether was administered were of different sexes, but this appears to be without influence upon the general result. In Experiment 4 more animals were employed and, considering their source, one would expect them to be of more uniform nature. The changes in the composition of the muscle and liver were smaller than in the previous experiments, but the variations were in the same directions.

In Experiment 5 animals from the same source as those used in Experiment 4 were employed. One is struck by the large individual variations in both control and experimental animals. They exceed the differences between the averages, and are so large as to raise some doubts as to the validity of drawing conclusions from the data. However, in the five experiments here reported, 78 animals in all were used and in all the experiments the variations observed were always in the same direction.

There is another way of regarding the data of Experiment 5. One would expect, for example, in those rats which showed the greatest change in the phosphorus content of the muscle compared with the control average to find the greatest increase in the liver

phosphorus as compared with the control average, and where the change is slight in the case of muscle tissue to find correspondingly little change in the liver phosphorus. When examined in this way the plausibility of the general conclusion is strengthened. Likewise, as a general rule, the muscle phosphorus of the control rats is greater than the liver phosphorus, but in the etherized rats this relation is reversed.

It is possible that the definiteness of the results obtained in this investigation would have been of a more decided nature had the phosphoric acid fractions been studied individually instead of *in toto*. It may be, for example, that only the inorganic fraction of the tissues undergoes change, though there is evidence that lecithin and lipoids generally, as well as fat, appear in increased quantity in the blood during ether narcosis (8, 9, 10).

The observation of Lange and Müller (11) that muscle fibers respond to long, deep narcosis by an increased elimination of phosphoric acid is in harmony with the results here presented.

The experiments just reported render inadequate the view that the increased phosphorus excretion observed in some of the simple ether experiments and in all the morphine-ether experiments was due to a discharge of phosphorus from one or more tissues. The data necessitate a different hypothesis concerning the cause of the lowered alkali reserve which develops during ether anesthesia. One sequence of events which appears somewhat probable is the following. During ether anesthesia phosphoric acid leaves the muscles unaccompanied by an equivalent amount of base (see data concerning base content of tissues in the rat experiments). This acid enters the circulation and partly neutralizes the alkali of the blood, thus lowering the alkali reserve. Because of depressed kidney activity the resulting alkali phosphates are not excreted while anesthesia endures, but are deposited; at least in part, in the liver, and only excreted or redistributed later when kidney function is more completely resumed.

The period in which this excretion or redistribution occurs has been mentioned in describing those experiments performed upon dogs with bladder fistulas. When ether alone is employed this takes place hours after the period of anesthesia, while in the case of morphine and ether the excretion begins promptly on beginning the ether and sometimes is ten times greater than the

rate of excretion of phosphorus in the control period. Concomitant with this increased excretion of phosphorus the urine always gave a positive Fehling test, due probably to the presence of dextrose; Fehling's test was positive in only one case of simple ether anesthesia. The prompt appearance of relatively large amounts of phosphoric acid in morphine-ether anesthesia we believe to be due to some interference with the capacity of the liver to hold the phosphorus which, as indicated by the rat experiments, is ordinarily deposited in the liver in simple ether anesthesia. This idea is supported by the glycosuria which occurs in the morphine-ether experiments and which may be due to some action of the morphine in exaggerating the glycemia of ether anesthesia by disturbing the carbohydrate metabolism of the liver.

In two instances we have determined the phosphorus content of the blood plasma during the control and morphine-ether periods, but, contrary to our expectation, found no increase. In a number of experiments in simple ether anesthesia we have also examined the blood before and after various durations of anesthesia and found, sometimes, increases and in other cases no change. The results are given in Table III. Brigg's modification of the Bell-Doisy method was employed.

Thus it is seen that the inorganic phosphoric acid content of the plasma increased in only about half of the experiments. However, it is evident that the phosphoric acid cannot be in the liver and in the blood as well, and failure to observe an increased content of inorganic phosphorus in the blood in all cases does not militate greatly against the view herein expressed.

Accompanying the increase in phosphorus content of the liver one might expect an increase in the sodium and potassium contents of the liver. However, the data of the rat experiments show no significant change in this regard. Of course, it is conceivable that the phosphorus in this case is associated with ammonia, but this does not throw any light on the whereabouts of that portion of the alkali metals which, before the anesthesia, was present in the blood as bicarbonate. There is a possibility of its having entered the corpuscles.

When no increase in the liver alkali was observed, assuming for the moment that free phosphoric acid entered the liver, it

was regarded as worth while to look for an increase in the hydrogen ion concentration of this organ. Two rats were used as controls and two were anesthetized with ether for 35 and 120 minutes, respectively.

TABLE III.

Dog No.	Anesthesia.	Inorganic P per 100 cc. plasma.
	<i>min.</i>	<i>mg.</i>
Control.		6.3
1	10	6.1
1	75	6.3
Control.		
2	20	5.5
2	120	8.9
Control.		3.8
3	60	6.8
3	120	7.4
Control.		4.4
4	120	4.9
4	180	5.2
Control.		2.9
5	30	6.7
Control.		7.4
6	60	6.8
Control.		5.7
7	60	5.8
Control.		5.9
8	40	6.4
Control.		5.9
9	60	5.9
Control.		4.0
10	165	6.7

In the case of each animal the liver and muscle tissues from the thighs were ground with finely divided Pyrex glass and the tissue was then dialyzed against distilled water for 20 minutes,

the contents of the collodion tube being stirred mechanically during the process. The determination was made electrometrically. At no time was any precaution taken to prevent the escape of CO_2 .

Control rate.		Ether rate.	
Muscle pH.	Liver pH.	Muscle pH.	Liver pH.
5.80	6.85	5.97	6.92
6.04	6.78	6.14	7.06

Instead of an increase in the acidity of the liver a decrease is exhibited. In the case of muscle tissue any change is doubtful though a decreased acidity is indicated, as one would expect, if phosphoric acid as such was discharged into the blood. Neglecting the failure to observe any increase in the base content of the liver by direct determination there is evidence in the pH measurements for such an accumulation. On the whole, however, it must be admitted that the problem of the whereabouts of the blood alkali during the anesthesia period is not elucidated in this communication.

CONCLUSIONS.

Data are presented which indicate that the excess of base excreted after a period of ether anesthesia is accompanied by an approximately equivalent quantity of phosphoric acid. The phosphoric acid appears to leave the muscles during the anesthesia and to sojourn in the liver until the reassumption of kidney function after the recovery of the animal when it is redistributed and partially excreted.

When morphine is administered as a preliminary to etherization a marked excretion of phosphorus occurs as soon as the ether is begun. This is attributed to an action of the morphine upon the liver which renders it incapable of retaining phosphorus.

It is suggested that the low alkali reserve and increased acidity of the blood in ether anesthesia is due to the discharge of phosphoric acid from the muscles.

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ON THE PREPARATION OF INSULIN.

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The procedure for the preparation of insulin outlined by us in an earlier abstract (1) was a modified form of the method first used successfully by the discoverers of insulin as described by Collip and coworkers (2). Retaining the use of alcohol for extraction the three important points added by us, and as a result of which great increases in yield and of purity were attained, were: (a) the use of much strong acid during the alcohol extraction, (b) the precipitation of the active substance from the crude aqueous solutions by ammonium sulfate, and (c) the precipitation of the insulin from the semipurified solutions by adjusting the reaction to pH 5 to 6.

The use of strong acid insures the solution of the insulin and prevents its destruction by proteolytic enzymes during extraction and subsequent evaporation. The precipitation by half saturation with ammonium sulfate accomplishes a complete separation from about nine-tenths of the accompanying proteins contained in the first extract and concentrates the activity in any desired volume. By precipitation of the insulin by adjusting the reaction to about pH 5, preparations were obtained of such activity that about 0.2 mg. of substance per kilo caused typical hypoglycemia and convulsions in rabbits weighing about 2 kilos. The yield of material of this or greater activity was by this procedure at least 500 "per kilo rabbit units" from each kilo of beef pancreas.

The purpose of this paper is to report the further purification of the product prepared by the method previously described

and as later improved, and to review the process of preparation in the light of the properties of the "insulin-protein." Since the activity expressed by the word "insulin," is (when obtained from beef pancreas at any rate) constantly associated with what appears to be a single, fairly well characterized protein, it becomes possible to formulate somewhat more rational methods for its extraction and purification than could be done before the physical and chemical properties of the product were known.

In consequence of the reviews of the literature contained in recent papers on this subject, we may omit detailed comment on other methods of insulin preparation. Attention may be called especially to the papers by Collip and coworkers, 1922 (2), Best and Macleod, 1923 (3), Murlin and associates, 1923 (4, 5), Best and Scott, 1923 (6), Witzemann and Livshis, 1923 (7), Dudley, 1923 (8), and Fisher, 1923 (9).

The "Insulin-Protein."

Although it would be premature to claim that our most active product is insulin, it behaves as an individual, fairly well characterized substance, which so far we have been unable to fractionate. Different preparations, purified by different methods, have the same properties and substantially the same degree of activity. Furthermore, we have not encountered active insulin preparations from which we were unable to isolate the characteristic substance. The last statement must be limited to preparations from beef pancreas; we have not used other material. For insulin of pancreas, the activity appears to be associated only with a single constituent. The purified insulin to which we refer is a protein, giving distinct biuret and Millon reactions, but negative glyoxylic acid reaction for tryptophane. Its activity is indicated by the fact that on injection into rabbits about 0.05 mg. (a conservative estimate) of dry substance per kilo of body weight causes convulsions and marked hypoglycemia, and on this basis about 0.03 mg. may be taken as 1 standard Toronto unit. (For a 2 kilo rabbit, $(0.05 \times 2) \div 3 = 0.033$ mg. for 1 unit.) In view of the above facts and of the rapid destruction of the activity by proteolytic enzymes (7, 8) we are

inclined to the view that the activity is a property of this protein, and not of a still more potent admixture.¹

If the view is correct that the activity is a property of "insulin-protein," it should be of great importance in affording a basis for the chemical assay of the substance. The present methods of physiological assay are tedious, difficult, and uncertain for quantitative purposes, and the ideal method is obviously the isolation and weighing of the active principle. Although we feel that our data alone are insufficient to establish the identity of insulin, we have been strongly impressed with the quantitatively very similar, if not identical, activity of our different "pure" preparations of "insulin-protein," purified by different methods, and with the fact that these different preparations appear to have otherwise the same properties.

As will be explained below we have separated from the "isoelectric insulin-proteins" described in our first report, two distinct proteins of less or no activity, and it is possible that the substance which we now call "insulin-protein" is still composed of more than one protein, only one of which is active. We have no evidence to disprove such an hypothesis. Whether or not insulin is identical with the "insulin-protein" can be determined only by the result of further efforts to separate and concentrate the activity.

The view above expressed (and suggested in our first report), that insulin is a protein, is in line with the conclusion of Dudley (8), but is contrary to the opinion first stated by Best and Macleod (3), and more recently by Murlin (5), who base their opinion that

¹ If insulin is not a protein and is present only as an admixture in purified "insulin-protein" it must have very high activity indeed. It could scarcely be present in amount more than 5 per cent of the weight of the "insulin-protein." That fraction of the quantity of "pure" "insulin-protein" which frequently gives convulsions in 1 kilo rabbits (0.01 to 0.02 mg.) would be 0.0005 to 0.001 mg. and this when distributed in the blood and tissues of the animal would give a concentration of the active principle of about 1 in 1 or 2 billion. This would be five to ten times the remarkable activity observed by Abel and coworkers (10) with their "pituitary tartrate," 0.01 mg. of which is required to produce rise of blood pressure on injection into cats. By the same sort of calculation the effective convulsive concentration of the "insulin-protein" itself would be of the order of 1 in about 50 million. Considering that it is a convulsive dose, either concentration expresses a very high degree of potency.

insulin is *not* a protein chiefly on the fact that some of their active solutions failed to give the biuret reaction. That evidence is, however, not necessarily conclusive for the reason that the test for activity considerably exceeds in delicacy the biuret reaction. Moderately pure preparations of "insulin-protein," diluted so that about 1 cc. is required to produce marked hypoglycemia and convulsions in rabbits (0.01 per cent or less of protein), are too dilute to show the biuret reaction. We are therefore inclined to suspect that the conclusions of Macleod and Best and of Murlin on this point have resulted from their use of too dilute solutions for the protein tests. In concentrations of 0.02 per cent (which corresponds to at least 5 "units" per cc.) or more, our purest (*i.e.* most active) preparations give an unmistakable biuret reaction, and according to analyses to be reported later by one of us, are beyond question protein in nature. Whether it is a derived protein or is present as such in the pancreas it is impossible at present to decide, though we incline to the latter view.

Much the most useful property of the "insulin-protein," for purposes of its separation from other substances and its purification, is its relative insolubility at a reaction of approximately pH 5, reported in our earlier communication (1). Following a partial purification of the crude extracts, this property affords an ideal separation, which has been adopted by the manufacturers (11, 12).²

In the absence of more than traces of salts, the range of its precipitation zone is between about pH 4.4 to 5.8, with optimum flocking out near pH 5, which doubtless represents approximately

² When this point was first communicated by one of us (P. A. S.) to Messrs. C. H. Best and D. A. Scott and, a few minutes later to Dr. G. H. A. Clowes, at Toronto, on December 28, 1922, it was unknown in the Toronto laboratory, though Dr. Clowes stated that it had been independently discovered in the laboratory of Eli Lilly and Co. (see outline of Lilly process, *J. Am. Med. Assn.*, 1923, lxxx, 1851). Recent examination (December, 1923) of two vials of "Insulin-Lilly," "H-10" from the stock of the Barnes Hospital, showed it to contain 0.07 mg. of solids (non-volatile at 110°C. and leaving no weighable ash on combustion) per labelled unit. The solids consisted of "insulin-protein," together with some of the "pH 4-proteins." These preparations were, therefore, according to our standards, of a high degree of purity, being about half as active as our best "insulin-protein".

its isoelectric point. It dissolves clear at pH 4 and at pH 6, and beyond this zone on either side, presumably in the form of acid and alkali salts.³

In the presence of even low concentrations of inorganic salts, especially sulfates, the range at which it precipitates is modified by being extended on the acid side. Solutions at pH 4 or at somewhat more acid reactions, which in the absence of salts are clear, are at once precipitated on the addition of small amounts of sodium or ammonium sulfate. Other salts have a similar, though less marked, effect and because of this effect salts must be removed for separation of the "insulin-protein" from the other "isoelectric proteins" to be described later. Alkali salts do not show such effects on the alkaline side of the isoelectric point, although at pH about 6 their presence may interfere with the separation just mentioned because of extending the precipitation zone of a less acid protein to be mentioned below. Moderate concentrations of salts have also the effect of slightly *increasing* the solubility of the "isoelectric proteins" within the precipitation zone.

Higher concentrations of salts, one-third to one-half saturation with ammonium sulfate and saturation with sodium sulfate or chloride, cause practically complete precipitation of the "insulin-protein" (and also of some other accompanying proteins) even at acid reactions far above its isoelectric range.

In alcoholic solutions, up to about 80 per cent, the purified "insulin-protein" is also quite soluble, but only in the form of its acid or alkali salts. At reactions near its isoelectric point, that is, in the form of isoelectric protein, it is little more soluble in dilute alcohol than in water. If a moderately strong aqueous solution is either slightly acidified with HCl or made alkaline with NaOH, or NH_4OH , it may be diluted with alcohol up to about 80 per cent without causing precipitation. From such clear alcoholic solutions the insulin is promptly precipitated on the addition of dilute alkali or acid to a reaction near the isoelectric point. On further increasing the alcohol concentration or adding ether, precipitation takes place even in the presence of an excess of acid, the completeness of precipitation depending upon the amount of acid excess as well as upon the alcohol concentration. If precipitated

³ As pointed out by Dudley (8), it is precipitated also by 3.3 per cent HCl. It is even less soluble in about 5 N H_2SO_4 than in HCl.

at pH 5 in aqueous solution, the addition of alcohol does not dissolve it. The activity is quite stable in the form of its acid salt and is less stable or unstable in the presence of excess alkali (7, 8).

Further information concerning the reactions and composition of the "insulin-protein" will be given in a later paper by Doisy and Weber.

Contaminating Isoelectric Proteins.—On adjusting the reaction to about pH 5, there separates from solutions of crude insulin (such as obtained by the Collip method or by dissolving in water the first half saturation ammonium sulfate precipitate) a precipitate which usually contains, in variable amounts, at least three proteins. One is the "insulin-protein" above described, and the other two are very similar in behavior except that each has, in the absence of salts, especially sulfates, a different range of precipitation, presumably due to different isoelectric points. All are precipitated at pH 5, especially in the presence of sulfates, contained in the solution referred to. One, which we term the "pH 8-isoelectric," has its optimum precipitation, in absence of salts, about pH 7 or 8 at which reaction both the "insulin-protein" and the third, the "pH 4-isoelectric," are quite soluble. Since low concentrations of alkali salts have little effect upon the solubility range on the alkaline side of the isoelectric points, the "pH 8-isoelectric" protein may be easily removed by adjusting the reaction with dilute ammonia to pH 7 or 8, at which reaction it precipitates, while the others remain in solution, even in the presence of some sulfates.

The purified "pH 8-protein" is not active on injection into rabbits. Different lots of the separated "pH 8-protein" have been injected in amounts from 0.17 to 4.3 mg. per kilo without causing symptoms or convulsions. (In a series of twelve rabbits, one which received 0.3 mg. had a convulsion after 3 hours.)

In the presence of sulfates, the ranges of precipitation of the "pH 4-isoelectric" and of the "insulin-protein" are so fused that their separation by fractional precipitation is difficult. But if both be precipitated together from such solutions at pH 5, the salt may be removed by washing by centrifugation several times with water at pH 5. On dissolving the combined precipitate in dilute acid (HCl or acetic) the "pH 4-isoelectric" precipitates on bringing the reaction to pH 4, at which reaction a large part of the "insulin-

protein" remains in solution. The substance which separates at pH 4 is, however, active, in all probability due to its carrying down some of the "insulin-protein." From such precipitates by repeated fractionation we are able to separate typical "insulin-protein," though we have not so far succeeded in removing all sugar lowering activity from the "pH 4-isoelectric" fractions. From the fact that such "acid" fractions appear to decrease considerably in their activity, we are inclined to suppose that the activity is due to contamination with the "insulin-protein."

We shall describe in a later section the procedures which we find preferable for the separation of the isoelectric proteins.

The Alcohol-Soluble Protein.

The aqueous solutions left after the evaporation of the alcohol from the first extracts of the pancreas hash, and after filtration from the fats and fatty acids, contain besides the "isoelectric proteins" described above, a protein which is in part precipitated with them by half saturation ammonium sulfate and is characterized by its ready solubility in alcohol up to about 80 per cent, and its insolubility in higher concentrations of alcohol. It is not precipitated from water or dilute alcohol on adjusting the reaction and thus may be separated from the "isoelectric proteins."

When present in relatively high concentration in solutions from which the "insulin-protein" is to be precipitated, it, however, considerably interferes with and renders incomplete that precipitation. This is often the case with solutions of the first $(\text{NH}_4)_2\text{SO}_4$ precipitate and especially with preparations obtained by the Collip method which consist chiefly of "alcohol-protein." (The success of the Collip method largely depends upon the fact that precipitation by strong alcohol of this "alcohol-protein" carries down with it varying amounts of the active "insulin-protein.") In such cases it is advantageous to reprecipitate once or twice by 0.4 saturation ammonium sulfate, which each time carries down substantially all the "insulin-protein" with progressively smaller amounts of the "alcohol-protein." In this way we have often obtained considerable amounts of "insulin-protein" of typical behavior and activity from solutions which failed to yield much precipitate on first adjusting the reaction.

If the relative amount of "alcohol-protein" is not too great simple dilution of the solution often increases markedly the yield of "isoelectric proteins."

The purified solutions of this "alcohol-protein" carry some activity, but its activity is relatively small and is not more than can be explained as due to the presence of traces of the "insulin-protein" which it is difficult or impossible to remove completely. It is not unlikely that this "alcohol-protein" is a group of proteins, and may include the toxic substance described by Fisher (9). It requires further study.

The Choice of Acid for Extraction.

As already noted, the use of much strong acid during the alcohol extraction of pancreas hash is essential to high yield. To this the Toronto workers agree (6). In our earliest preparations we added sufficient mineral acid (HCl , H_2SO_4 , H_3PO_4 , or HNO_3) to keep the alcoholic liquid acid to Congo red paper during the extraction. To this fortunate step is doubtless due the fact that from the first our preparations were remarkably active. The amounts of acid used were 10 to 40 cc. of 10 N for each kilo of pancreas. A large part of the acid is absorbed by the undissolved protein, with the formation of acid protein salts, and only by providing an excess can the desired reaction of the solution be secured. From the properties of the "insulin-protein" above described, it is now evident that to insure its solution the acidity should be safely above pH 4 and far above in the presence of salts. In order to secure a pH of about 3, at least 200 cc. of N acid are required for each kilo of pancreas. The buffer action of the proteins is so great that considerably more will be absorbed before the reaction becomes very strongly acid. In our earlier abstract we recommended 40 cc. of 10 N acid per kilo. This is unnecessarily great, half or three-fourths that quantity being sufficient and preferable. Less than this amount, according to our experience, is insufficient for good yield.

Table II contains data showing that the yield of activity with 2.5 cc. of 10 N H_2SO_4 was less (probably much less) than one-eighth as high as with 20 cc. of 10 N acid per kilo of hash. With the smaller amounts of acid only very small amounts of "isoelectric

proteins" and of activity were obtained; and with increasing amounts of acid the yield of "isoelectric protein" increased, parallel with the increase of activity. 20 to 30 cc. of 10 N acid per kilo of pancreas appear to be the optimum amount.

Of the common mineral acids, sulfuric is, according to our experience, the best. It causes minimum swelling of the hash with resulting rapid filtration and large volume of filtrate. Hydrochloric and phosphoric acids are objectionable because of their swelling effect, making filtration slow and yield of filtrate small. This difficulty can be in large part avoided by neutralization of the acid before filtration. Neutralization, however, greatly increases the salt concentration, and in theory, at least, endangers loss of insulin by precipitation. This danger can probably be avoided by neutralization to pH 7 or 8, on the alkaline side of the precipitation zone. But in this there is the practical difficulty that the combined acid of the undissolved acid protein salts is liberated slowly with consequent constant change of the reaction of the liquid. Furthermore, the solubility of other inactive proteins is changed on neutralization and extracts prepared with HCl (and alcohol), followed by neutralization, contain larger amounts of protein with corresponding difficulty in later purification of the active fraction. In spite of these objections successful preparations can be made with HCl, and in our earlier preparations with this acid the product was considerably lighter in color than those from the use of sulfuric acid. It is our impression, however, that the yield of activity with HCl, due to the necessity of neutralization, is apt to be lower than with H_2SO_4 . With suitable quantities of H_2SO_4 filtration is quite rapid without neutralization and by its omission not only may the danger of loss of activity by precipitation be avoided, but less of other contaminating protein is contained in the first acid extract and the whole process is considerably simplified.

Filtration without neutralization and the evaporation of the acid extract has another important advantage, besides those above mentioned. During the alcohol extraction a very considerable saponification of fat occurs, due doubtless to the lipase and the favorable effect of the alcohol on the solution and dispersion of the fat. If the extracts be evaporated at nearly neutral reaction, some of the fatty acid separates as alkali or protein

soaps which emulsify the remaining fat and proteins and thus make very troublesome their separation and filtration. But if the extracts remain rather strongly acid ($\text{pH} \pm 3$) during evaporation, the fat and fatty acids separate in a form which allows their subsequent complete removal by simple filtration through wet paper, and leaves the "insulin-protein" together with minimum admix-

TABLE I.

*Nitrogen Content of Pancreas Extracts and Insulin Preparations at Different Stages.**

Preparation No.	10 N acid per kg.	Alcohol used for extraction.	Filtered crude extract after evaporation of alcohol.	First $(\text{NH}_4)_2\text{SO}_4$ precipitate. Protein N.	"Isoelectric protein" precipitate.		Acidity of first crude extract after evaporation of alcohol (electrometric).
					mg. N	mg. solids	
	cc.	vol.	mg. N	mg. N			pH
94	20 HCl	1.5	3,950	273			
97	15 H_2SO_4	1.5	2,690	117	14	93†	
95	20 "	1.5	3,780	245	21	140†	
98	30 "	1.2	2,140	294	28	185†	
96	45 "	1.2	4,110		23	153†	
108	20 "	1.5	2,480	182	17	114†	
118	0	1.5					5.6
111 a	2.5 H_2SO_4	1.5	2,550				
111 b	5 "	1.5	2,840				5.0
111 c	10 "	1.5	2,410				4.4
111 d	15 "	1.5	2,250				3.5
111 e	20 "	1.5	2,090	182	8†	53	3.1
112	20 "	1.5	1,554	171	7†	48	3.1
113	20 "	1.5	1,554	157	5.4†	36	
114	20 "	1.5	3,400	230	10	67	
117	30 "	1.5	3,190		19	126	2.6
116	22 acetic.	1.5	3,600		Very small.		4.5

* Results expressed per kilo of pancreas hash.

† Calculated from nitrogen determinations, assuming 15 per cent nitrogen, or *vice versa*.

ture of other proteins in the perfectly clear filtrates. For this reason, if the first extracts are neutralized, they should be acidified (to Congo red paper) before evaporation. Unless the acidity of the solution at the time of filtration is pH 3 or greater, a considerable part of the "insulin-protein" is undissolved and is removed with the fat.

Table I contains data showing the reaction of the first extracts, after evaporation of alcohol and filtration from fat, for different amounts of acid used in the extraction of the pancreas hash. The extracts were made as described later (p. 43) except for the varying amounts of acids used. The determinations of pH were made by the electrometric method. It will be seen that when no acid is added, the reaction of the first crude aqueous extract is pH 5.6; with 5 cc. of 10 N H_2SO_4 , pH = 5.0; and with 10 cc. of 10 N acid, pH = 4.4. With 22 cc. of 10 N acetic acid, used by the Toronto laboratory as described by Best and Scott, the reaction was pH 4.5. All these reactions are within the zone of precipitation of the "insulin-protein," and as one would expect from this fact, the yield of "insulin-protein" and activity is with these amounts of acid relatively small. The "insulin-protein," obtained with the smaller amounts of sulfuric acid and with acetic acid, was not weighed, but from its volume on precipitation and centrifugation, was manifestly very small. This was the case even with 22 cc. of 10 N acetic acid.

The results, given in Table II, of injecting the crude extracts into rabbits, indicate the relative yield of activity from these preparations. An amount of extract equivalent to 4 gm. of pancreas per kilo of rabbit weight of the 2.5 cc. of 10 N acid preparation, to 2 gm. of the 5 cc. of 10 N acid preparation, and to 1.5 gm. of the 10 and 15 cc. of 10 N acid preparations, showed only little, although increasing activity, while an amount of isolated "insulin-protein" (0.026 mg.) representing only 0.5 gm. of pancreas of the preparation with 20 cc. of 10 N acid gave convulsions with blood sugar of 0.047 and 0.048. The pancreas used was from the same batch in all these preparations, and it is clear from the data that the amount of acid used, and the resulting hydrogen ion concentration, were the main factors responsible for the different yields. The low yield with small amounts of acid is probably due both to poor extraction from the pancreas and to precipitation during evaporation and loss with the fat on filtration. We did not determine separately the amount removed with the fat, and, therefore, cannot decide at which stage the greater loss occurs. Both during alcohol extraction and later filtration of aqueous extract the reaction was doubtless unfavorable for the solution of the "insulin-protein."

The other data of Table I concern the amount of nitrogen contained in extracts and fractions at different stages of the preparation. The first crude aqueous solutions after the evaporation of alcohol and filtration contain from 1.5 to 4.0 gm. of nitrogen per kilo of hash. Duration of extraction, concentration of alcohol, and the amount of acid added, all influence the amount of protein extracted. The total amount of protein extracted bears no constant relation to the yield of "insulin-protein" or of activity. The

TABLE II.

Illustrating the Effect of Amount of Acid Used for Extraction upon Yield of Activity.

Preparation No.	10 N H ₂ SO ₄ per kg. pancreas.	Rabbit No.	Weight of rabbit.	Amount injected per kg. rabbit weight.		Blood sugar.					
				Iso-protein.	Equivalent of pancreas.	1 hr.	1.5 hrs.	2.0 hrs	2.5 hrs.	3.0 hrs.	3.5 hrs.
			gm.	mg.	gm.						
111 a	2.5	74	3,270		4.0	No symptoms.				105	
111 b	5.0	400	3,600		2.0			79			
111 c	10.0	300	2,960		1.5			56			
111 d	15.0	70	2,770		1.0		62		100		
		35	2,860		1.5	78					144
111 e	20.0	36	2,720	0.078	1.5		43C				
		991	2,720	0.052	1.0		28	C	34	C	
		91	3,080	0.026	0.5		70	61		47	
		43	2,120	0.026	0.5		48C	C	C		
		80	1,800	0.022	0.4	51			60		
		60	2,550	0.01	0.2	63			95		

All prepared as described on page 43, except for different amounts of sulfuric acid in extraction of pancreas hash. C indicates typical hypoglycemic convulsions.

first crude extracts obtained by the procedure recommended in a later section yielding high activity, commonly contain 2 to 3 gm. of nitrogen; while as much or more nitrogen may be contained in extracts having only very little activity.

On precipitating the first crude extract by half saturation ammonium sulfate, the nitrogen content of the precipitate (after removal of ammonia by boiling with MgO and alcohol) is usually about one-tenth or less of the original, 0.15 to 0.25 gm. of nitrogen

per kilo of hash. The solution of this precipitate yields on adjusting the reaction to pH 5 (from successful preparations) about 0.05 to 0.16 gm. of total "isoelectric proteins," equivalent to about 0.007 to 0.025 gm. of N. By the successive use of ammonium sulfate and isoelectric precipitation, the activity is thus concentrated in less than 1 per cent of the material contained in the first alcoholic extract.

In this first precipitate the "insulin-protein" is contaminated with the accompanying "isoelectric proteins." When they are more or less completely removed, as later described, there should be left at least 0.05 gm. of fairly pure "insulin-protein" for each kilo of pancreas. The figures given in Table I for the amount of crude "isoelectric proteins," include for preparations Nos. 94 to 108, considerable "pH 4-" and "pH 8-isoelectric proteins;" while the amounts stated for later preparations, Nos. 111 to 117, represent the yield of fairly pure "insulin-protein" by extraction and precipitation of the first crude precipitate by buffers of known pH as described on page 45.

Taking 0.05 gm. as a conservative estimate of the amount of "insulin-protein" obtained from 1 kilo of beef pancreas, we may calculate that the content of "insulin-protein" in pancreas is 0.005 per cent.

Method of Preparation.

It may be stated in connection with the following description of the process for the preparation of insulin that we have used only common laboratory facilities, and have not had at our disposal high vacuum stills, continuous centrifuges, or other aids which are doubtless of importance for large scale production. Nevertheless, the process is smooth and simple and can be depended upon to yield without difficulty highly active preparations. (We are using it as a class exercise with medical students.)

Fresh beef pancreas is finely hashed by passing twice through a meat grinder. We commonly add 20 or 30 cc. of 10 N H_2SO_4 to each kilo of hash, and mix by stirring, before passing the second time through the grinder. If there is much delay in grinding, this is probably desirable. With a rapid motor-driven grinder it is not necessary. If the acid has already been added, add 1,500 cc. of 95 per cent alcohol: otherwise add that amount of alcohol and 20 or 30 cc. of 10 N acid per kilo of hash. Mix well

by stirring. After standing at room temperature for 4 to 12 hours, with occasional stirring, the mixture (without neutralization) is poured on large filters. The filtration is rapid, though it is convenient to allow it to drain overnight. The residue on the filters is pressed in a hand-press and the press-liquid filtered. A reextraction of the residue with 60 to 70 per cent alcohol, slightly increases the final yield. The combined filtrates (total without reextraction, about 2,100 cc. per kilo) are evaporated at low temperature. We have used a horizontal tunnel through which a current of warm air (40–45°C.) is blown by a motor fan, and in which the filtrates are placed in glass photographic development trays.⁴ The temperature of the liquid during evaporation in our apparatus is 25–30°C.

When the odor of alcohol is gone, and the volume reduced to one-tenth or less, the liquid is poured on moistened filter paper. (It is sometimes necessary to add water during evaporation to avoid too great concentration before all alcohol is gone.) Filtration is rapid and the separated fats and proteins are thus easily removed. The trays and filter are washed repeatedly with small amounts of water until the clear filtrate has a volume of about 200 cc.⁵ for each kilo of pancreas used.⁶

⁴ This apparatus does not provide for recovery of alcohol, which is blown to the outside air. (Its evaporation in a closed room caused an explosion.) Since only 1.5 to 2 liters of alcohol are used per kilo of pancreas, yielding 1,000 to 2,000 "units" of insulin, the alcohol, if tax-free or denatured, represents only a small item in the cost of production.

⁵ At this dilution the subsequent precipitation by ammonium sulfate carries down less "alcohol-protein" than when the extract is more concentrated. Because of its effect in interfering with the later precipitation of the "isoelectric proteins," it is desirable to remove most of the "alcohol-protein", and for this purpose a second precipitation with somewhat smaller concentration of $(\text{NH}_4)_2\text{SO}_4$ is advantageous.

⁶ The grayish brown precipitate on the filter, consisting chiefly of fat and fatty acid with some protein, contains also some "insulin-protein" which with high acid extraction is scarcely worth recovering. If recovery is desired the precipitate is well stirred with water acidified (Congo red paper) with HCl or H_2SO_4 and warmed to about 60°C. The mixture is then cooled in the ice box and filtered cold through moistened paper. The filtrate is precipitate with ammonium sulfate, the precipitate dissolved, and the isoelectric proteins are separated at pH 5, as described for the main fraction of the extract. The material recovered in this way appears to be less active than the main fraction, and we prefer not to combine them. Whether the lower activity is due to greater contamination or to injury of the insulin is undecided.

To the clear, although colored, filtrate (which has a reaction between pH 2.5 and 3.1) add 40 gm. of $(\text{NH}_4)_2\text{SO}_4$ for each 100 cc. and dissolve with stirring. On standing for some hours in the ice box the precipitate congeals and sticks to the walls and stirring rod, and allows the liquid to be poured off without loss. The brown gummy precipitate is dissolved in water and the liquid diluted to about 100 cc. for each kilo of pancreas hash, and is again precipitated by the addition of two-thirds its volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution. On standing some hours in the ice box the precipitate (much smaller in amount than before) congeals and sticks to the walls, the liquid is poured off, and if necessary, is centrifugated to avoid loss of small particles of the gummy material. The precipitate is dissolved in water with the addition of enough 0.1 N NH_4OH to make the reaction just distinctly yellow to methyl red (pH 6 to 8), which dissolves the "insulin-protein," but, if the reaction is not too alkaline, leaves undissolved any "pH 8-protein" which may accompany it. The solution is centrifugated and poured off from the dark colored precipitate, the latter being reextracted with water if desired. The combined solutions are diluted to about 100 cc. for each kilo of pancreas. On adding dilute acetic acid to about pH 5 (about midway of the color change of methyl red), a flocculent precipitate forms, which after standing some hours, is centrifugated out, washed with water at pH 5, and dissolved in a slight excess of 0.1 N HCl. From the mother liquor on standing for some days in the ice box, and by adding more acetic acid, more precipitate usually forms which is active, and is removed, dissolved, and added to the main fraction. This solution, although considerably colored and containing some admixed "pH 4-protein" is probably sufficiently pure for experimental or clinical use.⁷ From 50 to 100 mg. of this material are obtained from each kilo of pancreas hash, the larger amounts probably containing more of the "acid protein."

It is preferable to purify further the material as follows: The above mentioned precipitate, formed after adding acetic acid to about pH 5, after washing with water by centrifugation once

⁷ Prior to February, 1923, such preparations were used with diabetic patients in the St. Louis Children's and Barnes Hospitals, without evidence of any objectionable effect.

or twice to remove sulfates, is dissolved in a measured volume of ± 0.1 N acetic acid (5 or 10 cc. for each kilo of pancreas represented by the precipitate). Add exactly 20 per cent of the equivalent of NaOH. This mixture (acetic acid, one-fifth neutralized) gives a pH close to 4, at which reaction the "insulin-protein" is soluble while the "acid protein" in large part precipitates. After standing in the cold room some hours the precipitate is centrifugated out and washed by a second centrifugation with a smaller amount of water. (The buffer solution remaining in the precipitate suffices to maintain the pH of the wash water.) To the combined, often opalescent, supernatant solutions add an additional amount of NaOH corresponding to exactly one-half the equivalent of the acetic acid used. This gives a solution of acetic acid, seven-tenths neutralized, the pH of which is close to 5. At this reaction the "insulin-protein" separates at once and (after some days in the ice box if greatest possible recovery is desired) is removed by centrifugation, washed once or twice with distilled water, and dissolved in water by the addition of a few drops of dilute HCl.

The material obtained in this way, although of high activity or purity, probably contains some "pH 4-isoelectric" protein. Its solutions in slight excess of HCl are usually slightly opalescent, at pH 4 are dissolved but, not quite clear, and in fairly concentrated solution are more or less colored. By removing the "acid" fraction by precipitation at pH 4.3 instead of 4.0, the final solutions are less colored, and more nearly free from the "pH 4-protein," although the product gains but little in activity, at the cost of much loss of material. Repeating the fractionation at pH 4 is less costly. The activity of the buffer-purified "insulin-protein" is indicated by the data in Table III, from which it would appear that 0.03 to 0.05 mg. per kilo represents a unit dose; for a 2 kilo rabbit $(2 \times 0.03 \text{ or } 0.05 \text{ mg.}) \times \frac{1}{2} = 0.02 \text{ or } 0.033 \text{ mg.}$ as 1 standard Toronto unit. On this basis the yield may be estimated as from 1,500 to 2,500 "units" per kilo of pancreas.

By fractional precipitation from 80 to 95 per cent alcohol by addition of small amounts of acid or alkali, lighter colored (colorless) and apparently more active preparations have been secured. Table IV gives data to illustrate the activity of such alcohol-

purified preparations, from which it would appear that even 0.01 mg. per kilo is perhaps a unit dose. From the fact that such preparations were colorless and dissolved clear at pH 4 and 6, they were doubtless purer than those precipitated only from water, though we hesitate to conclude how much the activity was increased.

We are not disposed to urge the acceptance of these estimates of the amounts which represent a "unit," but prefer to leave the data to the reader's judgment. The great variability in the resistance of different rabbits to insulin has made its quantitative assay in our hands very uncertain. Only by closest attention in the selection and care of the rabbits, and by discarding those not in good condition, can fairly concordant results be obtained. Many of our animals were used repeatedly at intervals of a week or two, and at various times some had a respiratory infection ("snuffles") which seems to decrease resistance. All were fasted 24 hours before the injections. Rabbits fresh from the country are apt to be more resistant than after they have been kept for a time in the laboratory, even though they gain in weight and seem to be in good condition. In the face of these variations we venture no very exact statement as to how much "insulin-protein" represents a "unit," and are willing that the reader reach his own opinion from the data given in the tables. When, as above, definite quantities are mentioned in relation to units, it is to be understood that this qualification applies.

TABLE III.
Activity of "Insulin-Protein," Purified by Extraction at pH 7 and pH 4.*

Preparation No.	Rabbit No.	Weight.	Amount injected.		Blood sugar.							
			Total.	Per kg.	Be-fore.	1 hr.	1.5 hrs.	2.0 hrs.	2.5 hrs.	3.0 hrs.	3.5 hrs.	4.0 hrs.
			mg.	mg.								
79-2	11	2,670	0.84	0.31		C	G	C G				
	111	2,850	0.60	0.21				S		C G		
	1	2,110	0.42	0.20			G					
	5	2,190	0.40	0.18			C G					
	8	2,510	0.42	0.17			No symptoms.	No blood drawn.				
	321	2,650	0.42	0.16								
	989	2,480	0.40	0.16					C G			
	984	3,190	0.88	0.12				C G				
	991	2,370	0.29	0.12				C G				
	200	1,820	0.18	0.10				C G				
	992	3,150	0.31	0.10			No symptoms.	No blood drawn.				
	12	2,150	0.21	0.10					C G			
	29	2,560	0.25	0.10					C G			
	27	2,450	0.24	0.10								
	988	2,410	0.19	0.08		C						
	28	1,980	0.15	0.08				S				
	988	2,400	0.15	0.06	117	49	C	48 C	G			
	991	2,360	0.12	0.05	111	59		35 S		40 C		
	27	2,390	0.12	0.05		62	C	32	C			
	8	2,370	0.09	0.04		76		46				53

81		980	2,480	0.60	0.24	129	C G	65	58	56	45	55	72	105 (6 hrs.) 105 (5 ")
	1	2,240	0.40	0.18		118	C G	65	69	32 C	G			
	112	2,040	0.30	0.15		136	C G			70				
	321	2,750	0.25	0.09		112	C G							
	222	2,800	0.22	0.08										
	28	2,840	0.19	0.08										
	1	2,280	0.18	0.08										
	97	1,790	0.13	0.07										
	98	1,860	0.11	0.06										
	11	2,780	0.17	0.06										
	984	2,680	0.17	0.06										
	979	2,340	0.12	0.05										
	333	2,130	0.11	0.05										
	27	2,840	0.12	0.05										
	28	1,750	0.14			146		S	46	46 C		49	127 (6 hrs.)	
	988	2,300	0.11	0.05		143			69	62		72	107 (6 ")	
	8	2,340	0.12	0.05		148				41				
	321	2,850	0.17	0.06		111			56	49		67	120 (6 hrs.)	
									67		85	109	122 (5 ")	
	D 894	2,610	0.04	0.015					90					
	D 894	2,670	0.06	0.03				67		65				
	991	2,790	0.056	0.02				65	62					
	35	2,860	0.057	0.02				62	56					
	445	2,160	0.043	0.02				67	65					
112														

* C indicates convulsions; G, subcutaneous injection of glucose; and S, symptoms such as prostration or marked excitement. All blood determinations by the Shaffer-Hartmann method.

TABLE IV.
Rabbit Assay of "Insulin-Protein," Purified by Fractionation from Alcohol.*

Preparation No.	Rabbit No.	Weight. gm.	Amount injected.		Blood sugar.						
			Total mg.	Per kg. mg.	Before.	1 hr.	1.5 hrs.	2.0 hrs.	2.5 hrs.	3.0 hrs.	3.5 hrs.
D x 1	93	1,140	0.018	0.016		45 S		48		111	
	94	1,290	0.020	0.016		55	C	70	C		
	95	1,860	0.022	0.016	92	55	C	37			
	11	2,830	0.036	0.013		52		81	55	96	
	12	2,120	0.065	0.03		52	35				
	94	1,830	0.016	0.012		68		C			
	95	1,430	0.017	0.012		70		79			
	93	1,160	0.014	0.012		62		78			
	92	1,150	0.012	0.012		65	S	80			
	92	1,250	0.015	0.012		78 (?)		80		115	
	30	2,240	0.022	0.01		36	42		94		
	100	2,200	0.022	0.01		66	66		82		118
	29	2,710	0.027	0.01		67		47		76	
	27	2,600	0.026	0.01		50		52	80	101	118
D x 2	989	2,640	0.015	0.006		66	66				
	992	2,810	0.028	0.01		58		70	100	55	
D x 3	336	2,510	0.075	0.03		48		35			
	F	2,600	0.09	0.035		72			45 S		
	M ₁	2,620	0.09	0.034	104	50		30 C		40 C	44 C
	M ₂	2,750	0.09	0.033	120	110		64			70
	31	2,830	0.06	0.02	162	59	35 C	C			
	32	2,900	0.029	0.01		55	72		91		115
	1	2,450	0.015	0.006		83	58		83		108

*C refers to convulsions; S, to symptoms such as prostration or marked excitement; and G, subcutaneous injection of

TABLE V.

Summary of Assay of Some Early Preparations, Purified by Repeated Solution and Precipitation at about pH 5.

Preparation No.	Times reprecipitated from water solution at pH 5.	Injected mg. per kg. of rabbit weight.	No. of rabbits injected.	No. of rabbits showing:	
				Convulsions or blood sugar of 0.055 or lower.	No symptoms or blood sugars remained above 0.055.
55	Crude.	0.25 to 0.3	3	3	0
55 P	5	0.4	2	2	0
		0.2 to 0.25	9	9	0
		0.18	9	5	4
		0.13	2	0	2
		0.10 to 0.20	3	3	0
D 20	6 mos. later.	0.2 to 0.3	7	7	0
		0.15 to 0.19	14	11	3
		0.13	1	1	0
		0.2 to 0.25	4	3	1
59-1	10	0.2 to 0.25	4	3	1
60-1	4	0.16 to 0.28	7	4	3
	6 mos. later.	0.12 to 0.20	3	2	1
60-2	4	0.17 to 0.21	6	5	1

Undoubtedly contained "pH4-" and "pH 8-proteins" as well as "insulin-protein."

The following few examples will indicate the activity of different preparations. Table V illustrates the activity of the proteins separated by adjusting the reaction to about pH 5, of solutions prepared by the method outlined in our earlier abstract (1). The data given for Nos. 55 and 59 show that the activity of the isoelectric precipitate persists through its repeated solution and reprecipitation. The figures also appear to indicate that simple reprecipitation without a separation of proteins at particular reactions, accomplishes but little purification. On evidence of the sort contained in Table V, we based our earlier estimate of about 0.25 mg. per kilo as the rabbit unit. That estimate was probably overly conservative, for in a few cases the injection of much smaller amounts gave convulsions and very low blood sugar. The product from these early preparations undoubtedly contained

contaminating "isoelectric proteins." At the time these preparations were worked with, we had not clearly realized the existence of different proteins in the "isoelectric protein" fraction. Later preparations by improved methods showed activity from much smaller amounts, as illustrated in Table III, and we are disposed to believe that the difference is due in large part to smaller contamination with inactive "isoelectric proteins," and in part to our failure to inject smaller amounts of the early preparations. The products obtained by our improved method of preparation (which contain very little "acid" or "alkaline proteins") are smaller in amount as well as considerably more active than from the earlier method. It will be noted from Table I that while 140 mg. of crude "isoelectric proteins" were obtained per kilo of pancreas, representing perhaps 500 to 1,000 units, from the earlier preparations (No. 95), only 40 to 70 mg. are obtained with the same amount of acid by the process as now carried out (Nos. 111 to 114). The latter amounts represent, however, at least as great activity, 1,000 or more units, depending upon the amount taken as a "unit."

By purification of the early material, by fractionation from alcohol (as described under preparation No. 59), considerably more active fractions were obtained, as shown in Table IV. As little as 0.01 to 0.03 mg. per kilo fairly regularly gave convulsions and low blood sugar. By this method we have obtained our most active material.

Preparation No. 54 b.—Extraction by 1.5 volumes of 80 per cent alcohol and 40 cc. of 10 N HCl per kilo and neutralization before filtration. Residue after evaporation of alcohol, including fat, precipitated by half saturation with ammonium sulfate. The precipitate was extracted with 60 per cent alcohol acidified with HCl, filtered, and precipitated by strong alcohol. This precipitate was dissolved in water and the isoelectric protein separated by adjusting reaction to about pH 5. The solution "No. 54 b iso" contained 104 mg. of protein in 20 cc. for 1 kilo of pancreas hash. This solution was diluted and amounts varying from 0.1 to 1 mg. per kilo were injected into ten fasting rabbits, weighing 0.8 to 1.5 kilos, all of which had convulsions in from 1½ to 4 hours. Blood sugar was determined only in four in which cases it was 30 to 60 mg. after 3 hours. Since the smallest

dose gave convulsions, the per kilo dose is perhaps 0.1 mg. or less. Taking a more conservative estimate of double this amount the yield would be 800 units per kilo of pancreas, $(0.2 \times 2 \text{ for 2 kilo rabbits}) \div 3 = 0.13 \text{ mg. per "unit," } 104 \text{ mg.} \div 0.13 = 800 \text{ units per kilo.}$

Preparation No. 55.—Made in the same way as No. 54, 100 cc. of "isoelectric protein" solution contained 860 mg. of protein from 8 kilos of pancreas or 107 mg. per kilo. Of this 0.23 to 0.4 mg. per kilo injected into four 1 kilo rabbits gave convulsions in 2 or 3 hours, while 0.17 mg. in one rabbit gave no symptoms. Blood sugar was not determined.

This "crude" material was precipitated, dissolved, and reprecipitated with dilute NaOH and HCl five times, after which it ("No. 55 P") was injected into twenty-five rabbits with the results shown in Table V. It seems at any rate doubtful whether any increase in activity resulted from the purification. Doses of 0.13 mg. per kilo lowered the blood sugar to 79, but showed no symptoms; 0.18 mg. gave definite symptoms in five cases out of nine, but the blood sugar values are not very low, 39 to 60 mg. With 0.22 mg. and more the convulsions are more constant, and where determined the blood sugar was lower. About 0.2 mg. would be taken as the per kilo unit of activity.

6 months later the same solution was again injected into three rabbits, and even 0.1 mg. per kilo gave 49 mg. of blood sugar and convulsions. From this it might be supposed that the activity had increased, but it is more likely due to variations in susceptibility of the animals. The purified as well as the original solution doubtless contained a good deal of the "pH 4-" or "pH 8-protein," or both, which were carried through the "purification."

Preparation No. 59.—Made in the same way as No. 54, the crude total "isoelectric proteins" were precipitated, dissolved, and reprecipitated from water ten times. 0.3 mg. per kilo of the crude material caused convulsions in three rabbits, while about 0.2 mg. failed to cause symptoms in five rabbits. Blood sugar was not determined. From the "crude" solution a fraction was separated by precipitation at pH 6.5 to 6.8 which was later recognized as the distinct "pH 8-isoelectric" and which was inactive in amounts up to 0.37 mg. per kilo. (One rabbit receiving this amount had convulsions after 3 hours.) After re-

peated precipitation of the material freed from the "pH 8-protein," 0.2 to 0.25 mg. caused convulsions in three out of four rabbits. This material was considerably further purified by fractionation from alcohol as follows: It was again precipitated by addition of dilute alkali to about pH 5 and centrifugated. The precipitate was dissolved in 25 cc. of water by cautious addition of dilute alkali (pH 6 to 7) and 150 cc. of 95 per cent alcohol. Dilute HCl was then added cautiously first to opalescence and then to a flocculent precipitate. Judged by the color of methyl red the reaction was on the alkaline side of the optimum point of precipitation in aqueous solution. The solution was filtered after 1 hour in the ice box, and the precipitate (containing the "pH 8-protein"?) set aside. To the filtrate a little more acid was added, together with 100 cc. more alcohol, and the solution was placed in the ice box at 0°C. overnight. The precipitate which formed was removed by centrifuge, dissolved in acidified water, and reprecipitated twice, then dissolved in water with a few drops of dilute HCl. This solution, "No. 59 a P," contained 9.2 mg. per cc. and on injection into rabbits showed the activity indicated in Table IV.

To the alcohol mother liquor from which "No. 59 a" had separated, 1 drop of 0.1 N HCl and 100 cc. of ether were added. After 2 days in the ice box, a precipitate had formed which was removed by centrifuge, dissolved in acid water, and precipitated twice by cautious addition of dilute NaOH. A solution of the final precipitate was dissolved in water with addition of dilute HCl and labelled "No. 59 b P." The solution contained 3.6 mg. per cc. and after dilution was injected into rabbits as recorded in Table IV. Both Nos. 59 a and 59 b are quite active, a being more active than b. As little as 0.01 mg. per kilo of a caused convulsions and blood sugar of 40 after 1½ hours. The smallest dose injected of b, 0.03 mg. in one rabbit, caused symptoms in 3 hours, the lowest blood sugar being 0.058. The other preparations recorded in Table IV were purified by similar fractionation from alcohol.

SUMMARY.

A simplified method is described for the preparation of insulin from beef pancreas. The activity of insulin is probably a property of an individual protein, "insulin-protein," the solubility

of which is described. The method of extraction from pancreas and its purification is considered from the point of view of the properties of the "insulin-protein." It has been separated from two other proteins which also are precipitated at their isoelectric points, and which are included in the preparations of insulin, purified by "isoelectric precipitation," as earlier described by the authors.

Data are given to indicate the activity of the purified "insulin-protein."

Addendum.—A paper by Shonle and Waldo, from the laboratory of the Eli Lilly and Company, has just appeared (Shonle, H. A., and Waldo, J. H., *J. Biol. Chem.*, 1924, lviii, 731) reporting upon certain reactions and analyses of purified insulin preparations. They "conclude that the pancreatic substance containing insulin appears to be a complex mixture of proteoses from which it has been as yet impossible to isolate a simple substance." Of the eight different purified preparations cited by these authors, only one, No. 72,723, has about the same activity (0.0337 mg. per unit) as our better preparations, while most of their others are considerably less active. They report even more active material from a dialysate, 0.002 mg. of nitrogen of which corresponded to 1 unit. Assuming 18 per cent of nitrogen, this would represent 0.011 mg. of substance per unit, which is similar to the activity of our preparations purified by fractionation from alcohol. The table on page 734 indicates that all their other preparations contained very large proportions of ash (40 to 200 times as much ash as organic substance!) and this fact makes one suspect that these preparations, in spite of their purification by precipitation at isoelectric points, were contaminated with inactive isoelectric proteins and were far from "pure." They describe five of their most active preparations as having been purified by precipitation "at the isoelectric points or with trichloroacetic acid". We should not expect a general protein precipitant like trichloroacetic acid to be a favorable reagent for the separation of proteins having such similar behavior as have the isoelectric proteins of pancreas extracts.

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THE FORMATION OF ETHEREAL SULFATES.

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The work of earlier investigators such as that of Tauber (1), Kojo (2), and Sato (3), has shown that certain sulfur-containing compounds when fed along with phenolic bodies may increase the output of ethereal sulfates in the urine. Embden and Glaessner (4) have sought to determine the point where the ethereal sulfate synthesis takes place.

It seemed to us that the ethereal sulfate probably is not merely a combination of some aromatic hydroxy compound with sulfuric acid thus forming the ethereal sulfate at the expense of the inorganic sulfate, but that more than likely the ethereal sulfate is the result of the oxidation of a sulfur complex not unlike *p*-bromophenyl mercapturic acid (5, 6), and that this mercapturic acid found only in the urine of the dog (7) after feeding bromobenzene, represents an intermediary compound in the formation of the ethereal sulfate only found in the urine of other animals after feeding this benzene derivative.

To prove this point we decided to take the pig as an experimental subject, place him on a carbohydrate diet thus depriving him of all exogenous cystine, then feed not only bromobenzene but certain phenolic compounds known to combine with sulfuric acid, and then to study the connection between mercapturic acid formation and ethereal sulfate excretion.

The pig was chosen as the subject of the experiment, since this animal, as McCollum and Hoagland (8) have shown, can be maintained on a strictly carbohydrate diet long enough to secure and hold a condition in which the endogenous type of metabolism alone prevails. This is practically impossible with any other

animal, for loss of appetite, sudden collapse, or other serious disturbances so frequently upset the normal course of a prolonged experiment of this nature.

EXPERIMENTAL.

The animal selected was a healthy male of 24 kilos body weight. He was placed in a metabolism cage constructed in general according to the plan described by McCollum and Steenbock (9). The urine was collected at 24 hour intervals as naturally voided; *i.e.*, without catheterization. Throughout the work the daily diet consisted of 500 gm. of starch, with the following salt mixture: 1.5 gm. of potassium chloride, 5 gm. of sodium dihydrogen phosphate, 1 gm. of magnesium citrate, 6.75 gm. of sodium carbonate, and 0.75 gm. of ferric citrate. A diet of this type should keep the destruction of the body tissue down to a minimum. That this actually occurred may be seen from the nitrogen values in Tables I and II. The following determinations were made daily during the course of the work: total nitrogen, urea, ammonia, creatinine, uric acid, total sulfur, inorganic sulfates, and ethereal sulfates. The difference between the total sulfur and the sum of inorganic and ethereal sulfur gives the neutral sulfur figure. In the tables the only nitrogen values given are total nitrogen and urea, since ammonia, creatinine, and uric acid have little if any bearing on the interpretation of the sulfur figures. Moreover, all the sulfur values are presented in terms of *free* sulfur.

To avoid too great a strain upon the animal from the unusual length of the experiment, it seemed better to conduct the work in two parts. The results are given, therefore, in two tables, though otherwise there would hardly be need to separate the data. Preliminary to each experiment the pig was kept on the carbohydrate diet for several days, until the creatinine nitrogen comprised approximately 18 per cent of the total nitrogen. This condition can generally be reached with a healthy pig on a carbohydrate diet, and is in itself indicative of a state of minimal nitrogen metabolism.

The pig was then fed in turn phenol, bromobenzene, and *p*-chlorophenol and then each of these substances with varying quantities of first sodium sulfate, then later cystine, and lastly *p*-bromophenyl mercapturic acid was fed alone.

TABLE I.

Day.	Nitrogen.		Substances fed.	Sulfur values in terms of free S.				Sulfur percentages.		
	Total.	Urea.		Total.	Inorganic.	Ethereal.	Neutral.	Inorganic.	Ethereal.	Neutral.
	gm.	gm.		gm.	gm.	gm.	gm.	per cent	per cent	per cent
1	1.08	0.67		0.134	0.074	0.002	0.057	55	2	43
2	1.18	0.66		0.101	0.060	0.003	0.037	60	3	37
3	2.44	1.93	3 gm. cystine.	0.413	0.334	0.021	0.062	80	5	15
4	2.33	1.91	3 " "	0.476	0.409	0.019	0.047	86	4	10
5	2.06	1.56		0.203	0.132	0.006	0.066	65	3	32
6	1.00	0.68		0.156	0.107	0.006	0.040	69	4	27
7	1.14	0.82	2 gm. sodium sulfate.	0.553	0.497	0.011	0.044	90	2	8
8	1.29	0.84	2 " " "	0.657	0.571	0.020	0.066	87	3	10
9	1.30	0.79		0.321	0.251	0.013	0.057	78	4	18
10	1.17	0.72		0.109	0.072	0.005	0.032	66	5	29
11	2.43	2.37	0.6 gm. phenol.	0.206	0.097	0.045	0.063	47	22	31
12	1.66	0.98	0.8 " "	0.247	0.106	0.071	0.069	43	29	28
13	1.12	0.73		0.112	0.065	0.010	0.037	58	9	33
14	1.06	0.69		0.123	0.069	0.006	0.048	56	5	39
15	2.48	2.00	0.6 gm. phenol + 4 gm. cystine.	1.145	0.904	0.081	0.160	79	7	14
16	2.88	1.97	0.8 gm. phenol + 4 gm. cystine.	1.233	0.839	0.135	0.258	68	11	21
17	3.42	2.26		0.322	0.170	0.042	0.110	53	13	34
18	2.16	1.33		0.142	0.084	0.006	0.052	59	4	37
19	1.96	1.26	0.6 gm. phenol + 4 gm. sodium sulfate.	0.856	0.757	0.048	0.051	88	6	6
20	2.44	1.68	0.8 gm. phenol + 4 gm. sodium sulfate.	1.233	1.067	0.048	0.118	87	4	9
21	1.004	0.69		0.210	0.131	0.006	0.073	62	3	35
22	1.293	0.87		0.186	0.132	0.007	0.047	71	4	25
23	1.667	1.11		0.222	0.131	0.011	0.080	59	5	36
24	1.402	1.04	3 gm. <i>p</i> -bromophenyl mercapturic acid.	0.600	0.174	0.066	0.360	29	11	60
25	1.929	1.21	4 gm. <i>p</i> -bromophenyl mercapturic acid.	0.781	0.148	0.071	0.562	19	9	72
26	1.435	1.01		0.542	0.243	0.044	0.255	45	8	47
27	1.006	0.81		0.219	0.134	0.013	0.072	61	6	33

TABLE II.

Day.	Nitrogen.		Substances fed.	Sulfur values in terms of free S.				Sulfur percentages.		
	Total	Urea		Total	Inorganic	Ethereal	Neutral	Inorganic	Ethereal	Neutral
	gm.	gm.		gm.	gm.	gm.	gm.	per cent	per cent	per cent
1	1.09	0.68		0.127	0.067	0.005	0.055	52	4	44
2	1.12	0.71		0.106	0.064	0.004	0.038	60	4	36
3	0.97	0.66		0.087	0.042	0.004	0.042	48	4	48
4	1.01	0.64	2 gm. bromobenzene.	0.240	0.064	0.032	0.144	26	15	59
5	1.171	0.98	2 " "	0.313	0.113	0.060	0.140	36	19	45
6	1.16	0.59	2 " "							
			+ 3 gm. cystine.	0.744	0.452	0.027	0.265	61	4	35
7	1.46	0.83	2 gm. bromobenzene + 3 gm. cystine.	0.849	0.599	0.019	0.231	70	3	27
8	1.25	0.77	2 gm. bromobenzene + 2 gm. sodium sulfate.	0.950	0.734	0.017	0.199	77	2	21
9	1.16	0.59	2 gm. bromobenzene + 2 gm. sodium sulfate.	0.902	0.676	0.012	0.214	75	1	24
10	1.10	0.57		0.306	0.180	0.015	0.111	59	5	36
11	1.08	0.61	0.1 gm. chlorophenol.	0.312	0.141	0.025	0.146	45	8	47
12	1.23	0.72	0.2 " "	0.247	0.113	0.027	0.107	46	10	44
13	1.27	0.75	0.6 " "	0.227	0.095	0.041	0.091	42	19	39
14	1.69	0.99	0.6 " "							
			+ 4 gm. cystine.	1.254	0.730	0.022	0.502	58	2	40
15	2.03	1.19	0.6 gm. chlorophenol + 4 gm. cystine.	1.505	1.039	0.025	0.441	69	2	29
16	1.54	0.89	0.6 gm. chlorophenol + 4 gm. sodium sulfate.	1.662	1.337	0.020	0.265	82	1	17
17	1.32	0.87	0.6 gm. chlorophenol + 4 gm. sodium sulfate.	1.401	1.014	0.037	0.350	72	3	25
18	0.97	0.62		0.344	0.206	0.021	0.117	60	6	34
19	1.10	0.68		0.128	0.078	0.011	0.039	61	8	31
20	1.18	0.66	2 gm. bromophenyl mercapturic acid.	0.341	0.147	0.034	0.160	43	10	47
21	1.27	0.82	3 gm. bromophenyl mercapturic acid.	0.396	0.083	0.075	0.238	21	19	60
22	1.39	0.80	4 gm. bromophenyl mercapturic acid.	0.597	0.086	0.090	0.420	14	15	71
23	1.03	0.64		0.366	0.125	0.033	0.208	34	9	57
24	0.90	0.57		0.300	0.132	0.027	0.141	44	9	47

A comparison from the data of the various feedings reveals several interesting and important facts. The feeding of phenol caused the usual enormous increase in the output of ethereal sulfates; but when inorganic sulfate was given along with this same dose of phenol, instead of a further rise in ethereal sulfates there appeared a relative as well as a slight absolute drop. Again, the feeding of sodium sulfate simultaneously with *p*-chlorophenol seemed to have little effect, since practically the amount of ethereal sulfates was produced as when *p*-chlorophenol alone was ingested. Finally, the ethereal sulfate value following the administration of bromobenzene was decidedly *not* increased by the additional feeding of inorganic sulfates. In fact, a decrease was registered, just as in the former case of phenol plus sodium sulfate; and a quite marked decrease at that. There seems to be no manifest reason, however, for this drop, especially since the inorganic sulfates in both cases were excreted practically quantitatively in the urine. The results of the feeding of cystine with the aromatic poisons, however, are much more difficult to interpret. A moderate dose of phenol accompanied by more than enough cystine to supply sulfur for ethereal sulfate formation, resulted, as one would expect, in a doubling of the amount of ethereal sulfate excreted. But the administration of cystine with bromobenzene and with *p*-chlorophenol, cut the ethereal sulfate yield to one-half.

In the light of the results of the experiment, it is evidently not true to affirm that ethereal sulfates are formed simply at the expense of the inorganic sulfates. Indeed, inorganic sulfates do not seem to exert any positive influence in the matter at all. This much of a connection, however, may be admitted, that inorganic sulfates are formed from such exogenous and endogenous cystine as is *not* required for the production of taurine or ethereal sulfates. But even so, the formation of inorganic sulfates by the organism is apparently of very secondary consideration, in that ethereal sulfates receive first attention. It is quite probable that the elaboration of the ethereal sulfate molecule is begun from some intermediary form of cystine metabolism long before the sulfate stage is reached and that this conjugation product is later oxidized to a sulfate.

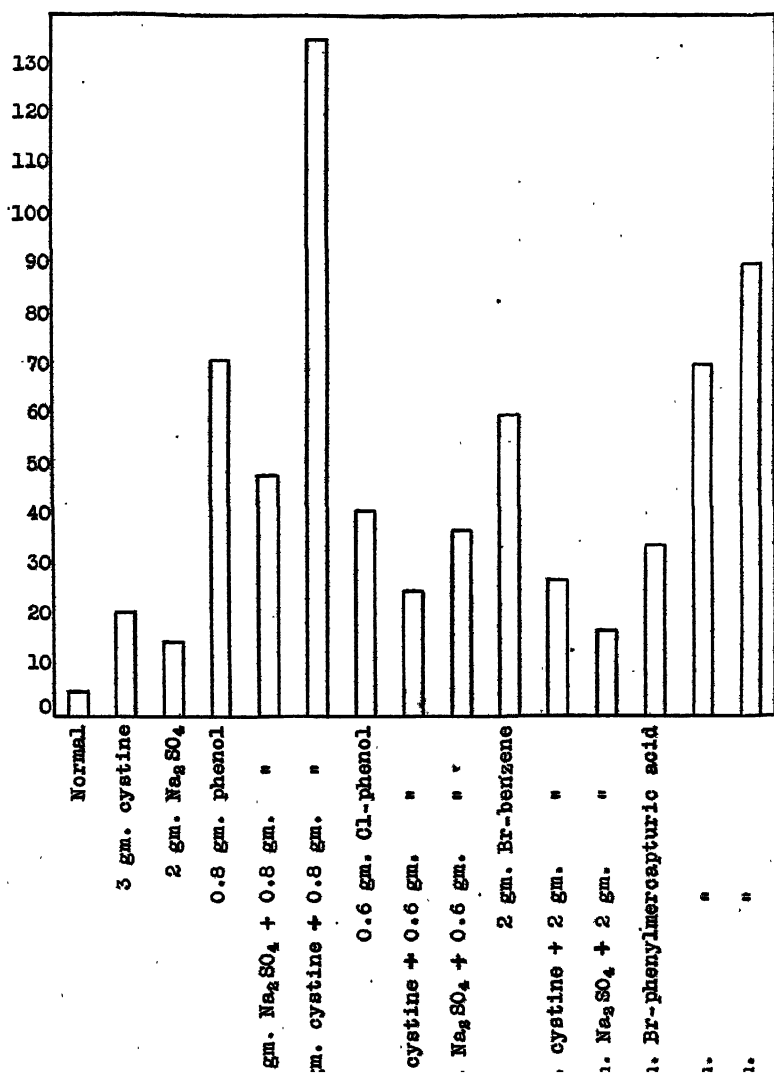


FIG. 1. Excretion of ethereal sulfates in milligrams per 24 hours.

with comparatively great difficulty in attempting this process on the corresponding compounds of cystine with bromobenzene and *p*-chlorophenol. Still, the assumption is not without strong support from other work conducted in our own laboratory (11). We found that when phenyl mercapturic acid, *viz.* $C_6H_5 \cdot S \cdot CH_2 \cdot CH(NH \cdot CO \cdot CH_3) \cdot COOH$, *p*-chlorophenyl mercapturic acid, and *p*-bromophenyl mercapturic acid were fed in equivalent amounts, as much as 58 per cent of the first compound was oxidized within 24 hours, while 43 per cent of the second, and only 23 per cent of the third suffered the same fate.

After we had completed this work, a paper appeared by Rhode (12), which corroborates in general our own results. He fed phenol to rabbits in doses of 0.2 gm. per kilo of body weight, and determined the ethereal sulfate excretion. He then repeated the feeding, but supplemented it with injections of the following substances: sodium sulfate, sodium sulfite, sodium thiosulfate, taurine, and cystine. He found that sodium sulfate and sodium thiosulfate exerted little, if any, influence on ethereal sulfate formation; but that taurine caused a moderate increase; sodium sulfite, a quite marked increase; and cystine, an increase of 33 per cent. He also fed bromobenzene and bromophenol, and observed in each case a rise in the output of ethereal sulfates. But when cystine was injected simultaneously with the bromobenzene or bromophenol, the ethereal sulfate excretion was reduced to about one-third of its former value.

SUMMARY.

A pig, reduced to a condition of endogenous nitrogen catabolism and maintained on a carbohydrate diet, excreted about 4 mg. of ethereal sulfates per day. The animal was then fed bromobenzene, phenol, and *p*-chlorophenol. The output of ethereal sulfates was very decidedly increased in each case, evidencing the formation of ethereal sulfates from endogenous sources. The feeding of inorganic sulfates along with each of these toxic substances resulted in *no increase* in the elimination of ethereal sulfates. Cystine, however, given together with each of the same aromatic poisons, caused an *increase* in the excretion of this form of sulfur in the case of phenol, but a *decrease* in each of the other two cases.

Moreover, in the case of the bromobenzene, the decrease was accompanied by a corresponding rise in the neutral sulfur fraction.

Apparently there are two ways of detoxicating phenolic substances; the one, by combining the poison with a sulfate radical, which is obtained by tissue destruction; the other, by utilizing exogenous cystine, forming eventually a mercapturic acid. This mercapturic acid may then be excreted as such, thereby adding to the neutral sulfur fraction and lessening that of ethereal sulfates, or it may be oxidized to a sulfate and increase the output of ethereal sulfates. This latter case was obtained when cystine and phenol were fed simultaneously.

It is believed that when ethereal sulfates are formed from endogenous sulfur, the toxic substance is joined to some intermediary product of the metabolism of tissue cystine, which product is not formed in the regular oxidation of exogenous cystine. This conjugate is then oxidized to a sulfate.

Our theory is confirmed by the fact that the mercapturic acids (*i.e.* acetylated cysteine derivatives) of phenol, *p*-chlorophenol, and bromobenzene, are subjected to oxidation by the organism to the extent of 58, 43, and 23 per cent, respectively, in 24 hours.

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GLYCOLYSIS IN BLOODS OF NORMAL SUBJECTS AND OF DIABETIC PATIENTS.

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Previous authors have disagreed as to whether the bloods of normal subjects and diabetics have different glycolytic powers. Although Lépine and his associates (1) firmly asserted that glycolysis in diabetic bloods is markedly diminished yet the preponderance of evidence obtained by others (2) definitely indicated that no significant difference exists between the glycolytic power of normal and of diabetic bloods. The latter view seemed the more accepted one, and from the following summary by von Noorden it appeared that the much disputed question was settled.

"There seems to be no doubt that the blood contains a sugar destroying ferment. Some maintain that this is identical with the widely distributed oxidizing ferment (Schmiedeberg, Jacquet, Spitzer, Salkowski) while others deny this (Jacoby, F. Blumenthal). No significant difference, however, has been proved to exist between the blood of diabetic and non-diabetic animals and men. Even in the most recent work upon glycolysis (Stoklosa, Šimacěk, Sieber, Feinschmid, Braunstein) there are such objections to the methods employed that the entire foundation of Lépine's researches seems to have collapsed."

With the advancement of newer theories regarding the causation of diabetes since the discovery of insulin, the question of glycolysis was reopened by Thalhimer and Perry (3) and Denis and Giles (4); both groups of workers maintaining that glycolysis proceeds at a slower rate in diabetic bloods.¹

¹Since this paper was submitted a very thorough and excellent study of glycolysis has come to my attention. This was reported by Bürger (Bürger, M., *Z. ges. exp. Med.*, 1923, **xxi**, 1, 98). Out of thirty-three cases the author finds complete inhibition in only one. He was a mild diabetic.

Because of such disagreement on this theme, apparently due to variation in technique, the study of glycolysis in normal men and diabetic patients was undertaken under uniform conditions.

The blood of eight normal subjects and eleven diabetic patients was studied. No breakfast was given in any case. 20 cc. of blood were drawn between 9 and 10 in the morning, and at once discharged into a sterile flask containing 40 mg. of potassium oxalate. A 2 cc. portion was withdrawn at once, and its sugar content determined. The remainder of the specimen was incubated at 37° C. and samples (2 cc. each) were taken for analysis after 1½, 3, 5, and 24 hours. Aseptic precautions were used. The sugar method of Folin and Wu (5) was used for all determinations, which were always done in duplicate. The red and white corpuscles were counted in each experiment and cultures were taken at the end of the 24 hour period from the original container.

The bloods of three normal subjects and of four diabetics were divided into two portions. One of these was kept at room temperature, while the other was incubated at 37° C.

Sugar determinations were carried out as above and at similar intervals, using aseptic technique. Cultures were also taken from the specimens which stood at room temperature.

From Table I and Fig. 1 it is clear that there is no difference in the glycolytic power of diabetic and non-diabetic bloods at 37°C. Corpuscular counts are also reported. These are within normal limits and within such limits bear no relationship to the rate of glycolysis. It is also apparent that although some of the bloods were contaminated at the end of the experiment, yet the loss of glucose from such specimens was no greater than of the ones which remained sterile.

Effect of Temperature.—At lower temperatures, differences have been reported. Chelle and Mauriac (6) found that at room temperature there was a definite lessening of glycolysis in diabetic bloods, but at 37°C. no difference in the glycolytic power of bloods of normal subjects and diabetic patients could be demonstrated. Denis and Giles (4) also reported that the rate of glycolysis was diminished in diabetic bloods. Their results were calculated on a percentage basis, and their experiments were done upon bloods kept at room temperature—two experimental procedures which have been criticized by earlier workers in this field.

TABLE I.

Sub- ject.	Sugar per 100 cc.					Loss per 100 cc.					White blood cells.	Red blood cells.	Culture.	Remarks.
	At once.	1½ hrs.	3 hrs.	5 hrs.	24 hrs.	1½ hrs.	3 hrs.	5 hrs.	24 hrs.					
		mg.	mg.	mg.	mg.		mg.	mg.	mg.	mg.				
T.	95.4	83.9	66.6		37.0	11.5	28.8		58.4	8,000	4,200,000	Not taken.	Normal.	
D.	96.1	74.0	66.9		20.0	22.1	29.2		76.1	7,400	5,030,000	Negative.	"	
L.	97.5	72.9	70.3	61.4	Trace.	24.9	27.2	36.1	90+	8,200	4,450,000	"	"	
R.	101.0	100.0	75.4	66.2	"	1.0	24.6	33.8	90+	7,600	4,840,000	Positive.	"	
B.	103.0	101.0	87.1	74.1	27.8	2.0	15.9	28.9	75.2	7,800	4,540,000	Negative.	"	
H.	255.6	240.9	212.7		Trace.	11.2	42.3		255+	7,000	4,000,000	Positive.	Diabetic.	
W.	149.8	127.6	106.0	96.8	39.3	22.2	42.8	53.0	100.5	7,400	5,000,000	Negative.	"	
D.	220.9	191.0	170.2	148.1	116.2	29.9	50.7	72.8	104.2	7,000	4,400,000	"	"	
M.	111.0	96.3	93.0	66.6	Trace.	14.7	18.0	44.4	105+	7,000	4,260,000	"	"	
C.	309.0	294.0	222.0	208.0	50.0	14.9	87.0	100.7	259.0	8,000	4,000,000	Positive.	"	
S.	375.0	375.0	316.0	303.0		0.0	59.0	72.0				Negative.	"	
K.	266.0	222.2	224.7	194.0	59.1	43.8	41.3	72.0	207.0	6,400	4,620,000	"	"	
R.	422.3	400.0	391.0	350.0	338.0	22.3	31.6	72.0	84.0	8,400	3,800,000	"	"	
L.	226.0	212.0	170.0	173.8	24.0	14.0	56.0	53.0	202.0	7,000	4,120,000	"	"	
G.	419.0	392.2	335.0	303.0	155.7	26.8	84.0	116.0	263.3	8,200	5,000,000	"	"	
M.	517.0	520.0	470.0	470.0	300.0		47.0	47.0	217.0			"	"	
													Coma. Died 18 hrs. later.	

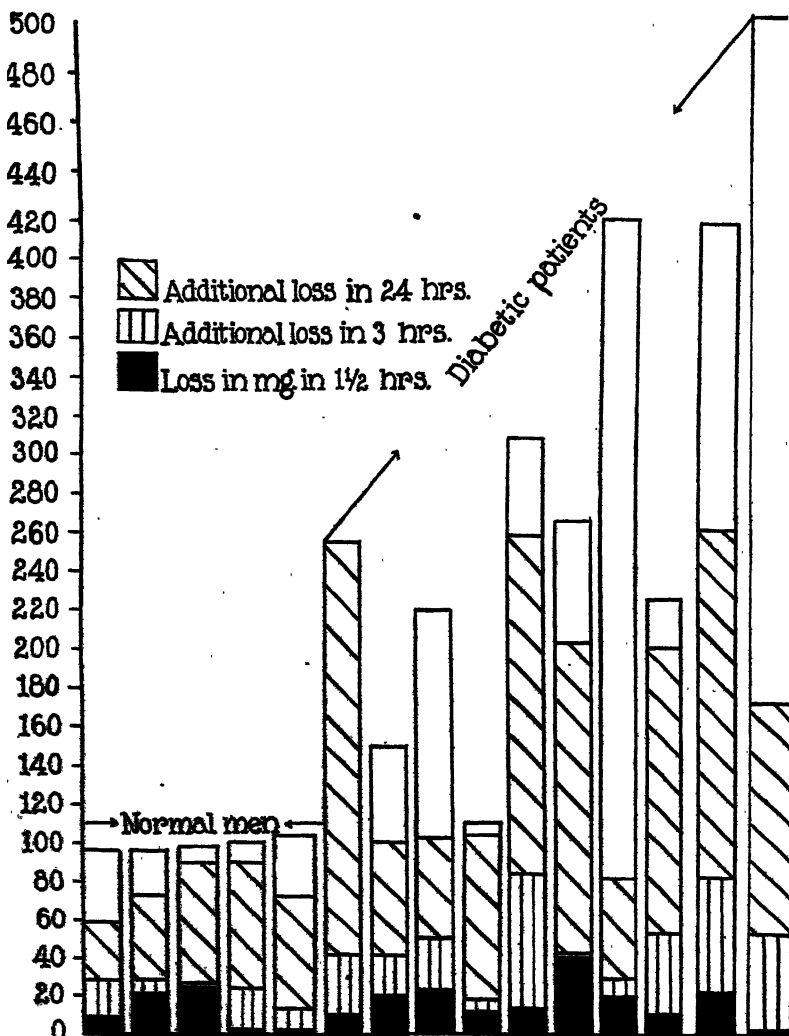


FIG. 1. Diagram showing glycolysis in normal and diabetic blood.

The height of the entire column represents the blood sugar immediately after withdrawal from the vein.

The loss in milligrams in the periods studied is represented by the various shadings.

TABLE II.

Subject.	Time.	Blood sugar per 100 cc.		Loss.		Remarks.
		At 37°C.	At room temperature.	At 37°C.	At room temperature.	
	<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
D.	At start.	95.2				Normal.
	1½	78.4	79.0	16.8	16.2	
	3	61.2	68.0	34.0	27.2	
	5	30.0	61.0	65.2	34.2	
	24	Trace.	20.0	90.0	75.2	
R.	At start.	102.0				Normal.
	1½	90.0	98.5	12.0	3.5	
	3	69.0	91.7	33.0	10.3	
	5	48.0	83.3	54.0	18.7	
	24	Trace.	44.0	100.0	58.0	
T.	At start.	95.4				Normal.
	1½	83.9	90.0	11.5	5.4	
	3	66.6	79.0	28.8	16.4	
	5		70.0		25.4	
	24	37.0	50.1	58.4	45.3	
C.	At start.	309.0				Diabetic.
	1½	294.0	288.4	15.0	20.6	
	3	222.0	250.0	87.0	44.0	
	5	208.0		101.0		
	24	50.0	188.6	259.0	120.4	
K.	At start.	266.0				Diabetic.
	1½	222.2	222.0	43.8	44.0	
	3	224.7	222.0	41.3	44.0	
	5	194.0	215.0	72.0	51.0	
	24	59.1	141.0	206.9	125.0	
L.	At start.	226.0				Diabetic.
	1½	212.0	210.5	14.0	15.5	
	3	170.0	188.5	56.0	37.5	
	5	173.8	188.5	52.2	37.5	
	24	24.0	130.0	202.0	96.0	
G.	At start.	419.0				Diabetic.
	1½	392.2	392.2	26.8	26.8	
	3	335.0	338.9	84.0	81.0	
	5	303.0	338.9	116.0	81.0	
	24	155.7	239.4	263.3	179.6	

That temperature influences the glycolytic rate has been known even at the inception of the study of this phenomenon. Arthus (7), who was among the first to study this relationship, clearly demonstrated, that he was able to recover as much as 80 mg. of glucose from a specimen which remained for 8 days at 10°C. and originally contained 133 mg., while at 40°C. the same specimen contained only 73 mg. at the end of 2 hours, and traces, of glucose at the end of 24 hours. In view of the fact that temperature is such a potent factor it is of extreme importance to subject the specimens studied to the same uniform thermal conditions, preferably a temperature comparable with that of the human body. Then, if any inferences are drawn from one's results, or hypotheses postulated, they will be of physiological significance. For, it seems unfair to perform a series of experiments at a temperature considerably lower than that which is the optimum for physiological processes, and then draw conclusions, that such results are at all comparable to processes which occur at a temperature definitely higher.

It was thought that the differences in results among the various workers on this subject might be explained by the fact that no uniform temperature conditions were employed. And, at first the work of Chelle and Mauriac seemed to offer the explanation; namely, that at room temperature the blood of diabetics glycolyzes more slowly, while no such difference is apparent at 37°C.

The results presented in Table II do not bear out this view. From the figures given, it is clear that the rate of glycolysis of diabetic and normal bloods is definitely slower at *room temperature* and that there is no great difference in the absolute amount of glucose lost in the two kinds of blood.

Another interesting feature, pointed out by Arthus (7) some time ago, is the relatively slow rate of glycolysis observed in the first 1½ hour period. Two of the *normal* bloods studied showed hardly any loss during this time.

SUMMARY AND CONCLUSIONS.

The glycolytic power of eight normal subjects and eleven diabetic patients was studied. Some of the bloods were studied

both at room temperature and at 37°C. Aseptic precautions were used and the red and white cells of the blood counted.

In the above series of observations, under the conditions described, no diminution in the glycolytic power of diabetic bloods was found when compared with that of normals when the bloods were kept at 37°C. It was also found that at room temperature the glycolytic rates were definitely diminished in both types of blood.

Bacterial contamination did not affect the results in the experimental period of 24 hours.

My thanks are due to Profs. Stanley R. Benedict and David P. Barr for their advice and interest throughout this work.

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EXTENSION OF THE VAN SLYKE TABLE OF FACTORS FOR THE CONVERSION OF NITROGEN GAS INTO MILLIGRAMS OF AMINO NITROGEN.

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(Received for publication, March 8, 1924.)

Van Slyke¹ has given a table of factors for the conversion of the volume of nitrogen gas into milligrams of amino nitrogen when the nitrogen is liberated in the determination of free amino groups by the Van Slyke¹ method. He says that the figures given in his table "are calculated by dividing by 2 those for moist nitrogen given by Gattermann in the *Praxis des organischen Chemikers*, ninth edition."

The table as given by Van Slyke and Gattermann includes only a rather narrow range of barometric pressure; namely, from 728 to 772 mm. of mercury. Many laboratories are located where the barometric pressure is much lower than 728 and thus it is necessary to calculate a new table. If the attempt is made to extrapolate the values given in the Van Slyke table to, for example, 630 mm., an error ranging from 1 to about 3 per cent may easily be made unless the table is subjected to a rather extended mathematical treatment. Van Slyke has shown that his method is capable of much greater accuracy than 1 to 3 per cent error. For the convenience of those who require factors corresponding to the lower barometric pressures a table has been calculated for intervals of 10 mm. over a range of barometric pressure from 520 to 780. The intermediate values can be interpolated by the use of the proportional parts given with the table. With the use of this table it is not necessary to go through the tedious process of calculating the values from the equation.

¹ Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.

The values given in the table were calculated according to the following equation:

$$\text{Factor} = \frac{(\text{Bar.} - \text{v.p. } H_2O \text{ at } t) \frac{(0.96727) (0.0012931)}{2}}{760 [1 + (0.003675 t)]}$$

Bar. refers to the barometric pressure in millimeters of mercury, at which the nitrogen gas was measured. From this value the vapor pressure of water at the temperature t , measured in degrees centigrade, is subtracted. The numerical values used in the equation were those obtained by the workers cited, as given by Landolt-Börnstein-Roth. The vapor pressure of water as determined by Scheel and Heuse² was used. The value 0.96727 is the density of nitrogen referred to air as unity obtained by averaging the values obtained by Lord Rayleigh, 0.96737, and Leduc, 0.96717.³ For the density of air the value 0.0012931⁴ was used. The product is divided by 2 because only one-half of the nitrogen is furnished by the free amino groups, the other half coming from the nitrous acid. The temperature coefficient, 0.003675,⁵ obtained by Chappius, was used.

¹ Landolt, H., Börnstein, R., and Roth, W. A., *Physikalisch-chemische Tabellen*, Berlin, 4th edition, 1912, 360.

² See foot-note 2, p. 150.

³ See foot-note 2, p. 18.

⁵ See foot-note 2, p. 350.

THE RATIO OF CARBON DIOXIDE TO HEAT PRODUCTION IN CATTLE.

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In the study of the metabolism of energy-producing feeds it is necessary to measure that extensive portion which expresses itself as heat.

The heat of metabolism may be measured directly, by means of a calorimeter, or indirectly, either from the energy value of the products of metabolism, or by computation from the carbon dioxide liberated, after the establishment, by direct calorimetry, of the ratio between the production of carbon dioxide and heat.

We have previously shown that a close relation exists between the CO₂ produced and the heat given off by cattle. The data expressing this relation, on a kilo of live weight basis, lend themselves to convenient expression by empirical linear equations of the general form $y = mx + b$. In these equations y may represent either Calories of measured heat or grams of CO₂ per kilo of live weight; m represents the slope of the line; x equals the air-dry weight of feed in grams per kilo of live weight; and b is the distance above the x -axis at which the line crosses the y -axis.

Any empirical equation really holds only for the data used in its deduction, and is subject to confirmation or change as additional data are secured, especially if these fall outside the range of variation of the old.

An earlier publication from this Institute¹ presented equations based on data from 99 experiments, 91 with steers and 8 with cows, the amount of air-dry feed varying from 5 to 27 gm. per kilo of live weight. These earlier equations were as follows:

¹ Armsby, H. P., Fries, J. A., and Braman, W. W., *Proc. Nat. Acad. Sc.*, 1920, vi, 263.

$$(1) \quad y_1 = 0.869 x + 14.176$$

$$(2) \quad y_2 = 0.455 x + 4.365$$

$$(3) \quad y_3 = -0.0226 x + 2.802, \text{ in which}$$

x = Air-dry weight of feed in grams per kilo of live weight.

y_1 = Calories of measured heat per kilo of live weight.

y_2 = Grams of CO₂ per kilo of live weight.

$$y_3 = \frac{\text{Heat}}{\text{CO}_2} = \frac{y_1}{y_2}$$

In these equations y_1 and y_2 are direct expressions of measured heat and carbon dioxide, while y_3 is deduced from a statistical treatment of all available individual ratios of y_1 to y_2 , and, therefore, may not be exactly equal, in every case, to y_1 divided by y_2 .

These equations apply to data involving feed consumption within the limits of 5 and 27 gm. per kilo of live weight. At the time these equations were computed there were no data available involving very low feed intake or obtained during fasting. Assuming, however, the applicability of the above equation to fasting, and putting x equal to zero, the carbon dioxide : heat ratio for fasting was predicted as follows:

$$\frac{y_1}{y_2} = 3.25 \text{ or } y_3 = 2.802^*$$

Since the time at which the above equations were computed, additional data have been secured at this Institute from 35 experiments with cows. Among these experiments were several in which the subjects fasted. The feed in these 35 cases varied from 0 to 25 gm. per kilo of live weight. The data were treated in precisely the same way as before, and empirical equations were computed. These equations agreed closely with equations (1), (2), and (3). All data were then combined in a new set of equations representing the 134 experiments, 91 with steers and 43 with cows. The new equations are the following:

$$(A) \quad y_1 = 0.776 x + 15.061$$

$$(B) \quad y_2 = 0.420 x + 4.759$$

$$(C) \quad y_3 = -0.02886 x + 2.883$$

* The fact that y_3 in equation (B) does not equal $\frac{y_1}{y_2}$ indicates, as was noted in the previous article, that y_2 is not strictly a straight line function of the amount of feed. It was then suggested that $\frac{y_1}{y_2}$ was the better form to use with very small amounts of feed.

These differ but slightly from equations (1), (2), and (3). The slope of the line representing the ratio (y_3) is slightly increased by the inclusion of the data representing the fasting experiments.

When x is made equal to zero, in the new equations, and y_1 is divided by y_2 , we have $\frac{y_1}{y_2} = 3.165$. Assigning to x the value 0 in equation (C), $y_3 = 2.883$. These two values for y_3 are more closely in agreement than similar values derived from the earlier equations, and they show that the prediction of the CO_2 : heat ratio for fasting was approximately correct.

Chart I shows curves of heat and carbon dioxide production and of the ratio of the heat to the carbon dioxide in each individual case.

These equations hold for resting animals, doing no external work, and lying or standing at will.

It is well known that both the CO_2 and the heat produced are greater while the animal is standing than while lying. However, the ratio CO_2 : heat is not much affected. In the previous paper, the range of the ratios from 51 experiments varied, while standing, from 1.86 to 2.94, and while lying from 1.69 to 2.57, showing a large overlapping of the two.

The later experiments show wide variation in relative time spent standing and lying, but no related variation in CO_2 : heat ratios. Therefore, no account has been taken of the change in position of the animals in these experiments. The number of experiments is large, and the assumption is made that the results hold for average conditions as to standing and lying.

Applying equation (C) to our data, and computing the heat from the observed CO_2 , we get results as set forth in Table I. In the last column is given the percentage of observed heat on the basis of the heat computed by use of the ratio. All but 35 of the 134 cases fall within the range of 95 to 105 per cent.

The fact that these percentages, in the case of the fasting animals, all fall considerably above 100 signifies that the mathematical equation should indicate a line slightly curved in that portion representing very low feeding, or fasting.

Something should be said regarding these periods. Experiments 221 D, Cow 886 IV, 1st day, and 221 D, Cow 886 IV, 2nd day, were succeeding days with the same animal, which had

TABLE I.
Comparison of Computed and Observed Heat Production.

Experi- ment No.	Animal.	Feed per kilo live weight.	Observed CO ₂ produced.	g ₂ ratio.	Heat produced computed by ratio.	Observed heat produced.	Observed + com- puted.
		gm.	gm.		Cals.	Cals.	per cent
221 D	Cow 886 IV (1st day)	0	2,223	2.883	6,408	6,743	105.2
221 D	Cow 886 IV (2nd day)	0	1,987	2.883	5,728	6,328	110.5
	Cow 885 IV (1st day)	0	2,247	2.883	6,479	6,750	104.2
	Cow 885 IV (2nd day)	0	2,148	2.883	6,193	6,557	105.9
221 E	Cow 885 III	0	2,034	2.883	5,864	6,577	112.2
221 F	" 887 III	0	1,885	2.883	5,434	6,061	111.5
	" 874 III	0	2,091	2.883	6,030	6,302	104.5
211 V	Steer D	4.68	2,808	2.748	7,716	7,953	103.1
211 V	" G	4.94	2,437	2.740	6,678	6,882	103.1
186 I	" A	6.06	3,957	2.708	10,715	10,911	101.8
186 I	" B	6.06	4,077	2.708	11,040	11,736	106.3
220 V	" K	6.11	3,875	2.707	10,490	10,060	95.9
207 III	" A	6.70	3,095	2.690	8,326	7,775	93.4
209 III	" F	6.77	2,316	2.688	6,226	6,354	102.1
208 III	" E	6.87	1,784	2.700	4,816	4,906	101.9
208 III	" C	6.96	2,223	2.682	5,963	5,948	99.7
179 II	"	7.12	3,682	2.678	9,859	10,123	102.3
200 III	" A	7.52	2,803	2.666	7,472	7,431	99.5
211 II	" G	7.55	3,203	2.665	8,536	8,197	96.0
216 IV	" J	7.86	3,134	2.656	8,323	7,967	95.7
208 VI	" E	8.14	1,663	2.648	4,403	4,648	105.6
190 III	" A	8.37	2,265	2.641	5,981	6,007	100.4
186 III	" A	8.45	4,218	2.639	11,133	10,724	96.3
186 III	" B	8.45	4,162	2.639	10,985	10,874	99.0
207 III	" B	8.56	3,070	2.636	8,094	7,897	97.6
209 VI	" F	8.57	2,475	2.636	6,525	6,722	103.0
208 VI	" C	8.72	2,199	2.631	5,785	5,937	102.6
211 IV	" D	8.80	3,542	2.629	9,312	9,196	98.8
221 E	Cow 885 I	8.84	3,940	2.628	10,354	9,788	94.5
179 III	Steer	8.85	4,048	2.628	10,638	10,540	99.1
221 E	Cow 885 II	8.91	3,515	2.626	9,232	8,715	94.4
220 II	Steer K	9.05	3,926	2.622	10,294	10,291	100.0
220 III	" K	9.19	4,426	2.618	11,587	10,754	92.8
217 IV	" J	9.23	5,894	2.617	15,425	14,225	92.2
211 II	" D	9.25	3,889	2.616	10,175	9,596	94.3
208 II	" D	9.26	1,465	2.616	3,832	4,023	105.0

TABLE I—Continued.

Experiment No.	Animal.	Feed per kilo live weight.	Observed CO ₂ produced.	$\frac{1}{2}$ s ratio.	Heat produced computed by ratio.	Observed heat produced.	Observed + computed.
		gm.	gm.		Cals.	Cals.	per cent
211 IV	Steer G	9.31	3,357	2.614	8,775	8,936	101.8
200 III	" B	9.45	2,592	2.610	6,766	6,913	102.2
208 II	" C	9.48	2,684	2.609	7,002	7,048	100.7
210 III	" D	9.51	2,716	2.609	7,085	7,106	100.3
179 I	"	9.54	4,351	2.608	11,346	11,529	101.6
208 II	" E	9.91	2,129	2.597	5,529	5,525	99.9
221 D	Cow 885 I	10.24	3,361	2.587	8,695	8,378	96.4
186 II	Steer A	10.28	4,619	2.586	11,946	11,435	95.7
186 II	" B	10.28	4,538	2.586	11,736	11,318	96.4
190 III	" B	10.30	1,905	2.586	4,926	5,212	105.8
221 D	Cow 886 III	10.31	3,409	2.585	8,812	7,891	89.5
212 V	Steer H	10.38	2,851	2.583	7,364	7,305	99.2
217 I	" J	10.40	4,634	2.583	11,969	10,895	91.0
221 D	Cow 885 III	10.43	3,696	2.582	9,543	8,814	92.4
216 VII	Steer J	10.61	3,253	2.577	8,317	8,229	98.9
212 VI	" H	10.63	2,695	2.576	6,941	6,773	97.6
207 IV	" A	10.89	4,055	2.569	10,417	9,493	91.1
209 II	" F	10.90	2,903	2.568	7,455	7,427	99.6
221 D	Cow 886 I	10.96	3,440	2.567	8,830	8,034	91.0
221 F	" 874 II	10.98	3,809	2.566	9,773	9,077	92.9
216 II	Steer J	11.46	3,909	2.552	9,977	9,533	95.5
207 I	" A	11.50	4,354	2.551	11,106	10,171	91.6
174 I	" B	12.26	3,944	2.529	9,975	9,215	92.4
200 IV	" A	12.30	3,353	2.528	8,476	8,183	96.5
221 G	Cow 887 II	12.59	3,451	2.520	8,696	8,626	99.2
200 I	Steer A	12.62	3,832	2.519	9,652	9,307	96.4
208 V	" C	12.67	2,732	2.517	6,876	7,117	103.5
210 II	" D	12.75	3,236	2.515	8,140	8,186	100.6
221 F	Cow 887 II	12.81	3,522	2.513	8,850	8,396	94.9
209 V	Steer F	12.97	3,011	2.509	7,555	7,673	101.6
207 I	" B	12.98	3,924	2.508	9,840	9,537	96.9
208 V	" E	13.02	2,190	2.507	5,490	5,697	103.8
220 I	" K	13.63	4,841	2.490	12,055	12,124	100.6
200 IV	" B	13.77	3,934	2.486	9,779	9,428	96.4
221 C	Cow 885 III	13.92	3,608	2.481	8,952	8,855	98.9
200 IV	Steer B	13.93	2,999	2.481	7,440	7,359	98.9
200 I	" B	14.09	3,561	2.476	8,816	8,907	101.0
190 I	" A	14.22	3,201	2.473	7,915	8,589	108.5
221 C	Cow 885 II	14.30	3,729	2.470	9,212	8,862	96.2
221 D	" 885 II	14.34	4,068	2.469	10,043	9,623	95.8
190 IV	Steer A	14.41	2,907	2.467	7,171	7,344	102.4

TABLE I—Continued.

Experiment No.	Animal.	Feed per kilo live weight.	Observed CO ₂ produced.	$\frac{1}{2}$ ratio.	Heat produced computed by ratio.	Observed heat produced.	Observed ÷ computed.
		gm.	gm.		Cals.	Cals.	per cent
179 IV	Steer	14.47	6,138	2.465	15,130	14,652	96.8
221 C	Cow 886 II	14.55	3,599	2.463	8,864	8,568	96.7
220 IV	Steer K	14.61	5,949	2.462	14,647	13,838	94.5
208 I	" D	14.66	1,941	2.460	4,774	5,031	105.4
174 II	" C	14.81	4,368	2.456	10,728	10,296	96.0
210 I	" D	14.82	3,812	2.455	9,358	9,450	101.0
211 I	" D	15.23	4,824	2.443	11,786	11,547	98.0
216 III	" J	15.37	4,725	2.439	11,524	10,811	93.8
221 F	Cow 874 I	15.64	4,882	2.432	11,872	11,099	93.5
190 IV	Steer B	15.82	2,193	2.426	5,320	5,424	101.9
190 I	" B	16.11	2,593	2.418	6,271	6,610	105.4
221 D	Cow 886 II	16.25	4,773	2.414	11,521	10,516	91.3
207 II	Steer B	16.43	4,899	2.409	11,802	11,517	97.6
212 III	" H	16.96	4,054	2.394	9,705	9,723	100.2
200 II	" B	17.12	3,964	2.389	9,469	9,602	101.4
212 IV	" H	17.17	4,004	2.387	9,558	9,701	101.5
207 II	" A	17.35	6,268	2.382	14,930	14,130	94.6
216 VI	" J	17.36	4,569	2.382	10,882	10,829	99.5
174 IV	" D	17.51	4,730	2.378	11,247	11,493	102.2
208 I	" E	17.85	2,928	2.368	6,933	7,085	102.2
209 I	" F	17.92	3,840	2.366	9,087	8,975	98.8
211 I	" G	17.98	4,839	2.364	11,440	11,711	102.4
217 III	" J	18.09	9,074	2.361	21,423	21,196	98.9
221 F	Cow 887 I	18.21	4,549	2.357	10,722	10,324	96.3
200 I	Steer A	18.78	5,511	2.341	12,902	12,827	99.4
190 II	" A	18.81	3,726	2.340	8,719	9,684	111.1
208 IV	" C	19.05	3,573	2.333	8,335	8,976	107.7
221 G	Cow 887 III	19.05	4,309	2.333	10,054	9,868	98.2
211 III	Steer D	19.17	6,216	2.331	14,490	13,937	96.2
217 II	" J	19.32	7,509	2.325	17,458	16,660	95.4
221 E	Cow 874 II	19.32	5,023	2.325	11,678	11,514	98.6
221 E	" 886 II	19.76	4,948	2.313	11,445	11,027	96.3
221 C	" 885 I	19.82	4,948	2.311	11,435	11,227	98.2
221 E	" 874 I	20.14	5,031	2.302	11,582	11,412	98.5
190 II	Steer B	20.31	2,891	2.297	6,412	7,303	113.9
211 III	" G	20.37	5,940	2.295	13,633	13,440	98.6
221 A	Cow 631 III	21.30	5,828	2.268	13,219	13,219	100.0
212 I	Steer H	21.51	4,719	2.262	10,675	11,023	103.3
212 II	" H	21.51	4,593	2.262	10,390	10,904	104.9
221 C	Cow 886 I	21.61	4,889	2.259	11,045	11,193	101.3
221 F	" 886 I	21.66	5,740	2.258	12,961	13,583	104.8

TABLE I—*Concluded.*

Experiment No.	Animal.	Feed per kilo live weight.	Observed CO ₂ produced.	$\frac{1}{2}$ ratio.	Heat produced computed by ratio.	Observed heat produced.	Observed ÷ com- puted.
		gm.	gm.		Cals.	Cals.	per cent
221 F	Cow 886 II	21.96	5,573	2.249	12,534	12,333	98.4
221 F	" 886 I	22.14	5,466	2.244	12,267	11,932	97.3
208 IV	Steer E	22.29	3,074	2.240	6,886	7,686	111.6
216 V	" J	22.29	5,505	2.240	12,381	12,739	103.3
221 C	Cow 886 III	22.42	5,013	2.236	11,208	11,065	98.7
221 G	" 887 I	23.01	5,453	2.219	12,099	12,393	102.4
221 A	" 631 II	23.16	5,518	2.215	12,221	12,334	100.9
221 A	" 615 III	23.37	5,447	2.209	12,032	12,032	100.0
221 A	" 615 II	23.51	5,332	2.205	11,757	12,137	103.2
221 A	" 615 I	23.92	5,146	2.193	11,285	11,435	101.3
221 G	" 887 IV	24.61	5,243	2.173	11,393	11,652	102.3
209 IV	Steer F	24.93	4,424	2.164	9,573	11,015	115.1
221 A	Cow 631 I	25.00	5,156	2.161	11,141	11,464	102.9
221 A	" 579 II	25.58	5,834	2.145	12,513	12,896	103.1
221 A	" 579 I	25.94	5,931	2.134	12,657	13,236	104.6
216 I	Steer J	27.01	7,045	2.103	14,817	15,534	104.8

fasted just 24 hours before the beginning of the 1st day. The considerable amount of methane given off, and the reduction of that amount from 27.4 gm. on the 1st day to 11.8 gm. on the 2nd day, indicate that the post-resorptive state had not been reached. The same is true with No. 221 D, Cow 885 IV, 1st and 2nd day, the methane decreasing from 33.5 gm. on the 1st day to 17.4 gm. on the 2nd day.

With No. 221 E, Cow 885 III the CO₂ and heat measurements were made after a preliminary fasting of 3 days, and continued for 2 days. On the 1st day the methane outgo was 6.48 gm., and on the 2nd day, 5.06 gm., indicating a nearer approach to the fasting condition.

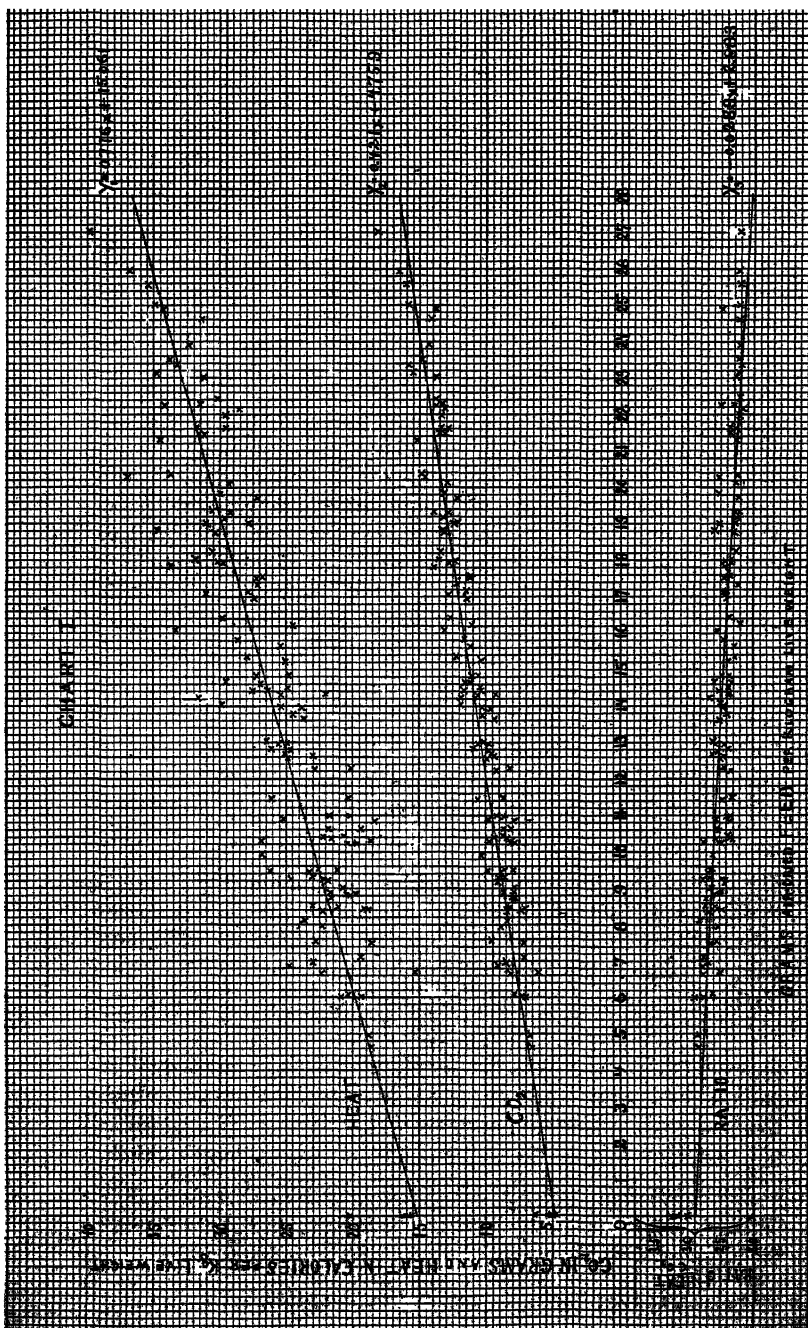
In Experiments 221 F, Cow 887 III and 221 F, Cow 874 III the preliminary fasting lasted 7 days, and the methane for the 2 succeeding days, in the one case, was 2.75 and 2.92 gm., respectively, and in the other, 4.15 and 4.09 gm., indicating a nearly constant minimum, and a condition of fasting.

It is interesting to apply this ratio to some of the earlier experiments in which the respiration was determined, and the heat

TABLE II.

Comparison of Computed and Observed Heat Production Computed from Kellner's Data.³

Animal.	Experiment No.	Feed per kilo live weight.	Observed CO ₂ produced.	ys ratio.	Heat produced computed by ratio.	Heat produced computed indirectly.	Indirect computed ÷ ratio computed.
		gm.	gm.		Cals.	Cals.	per cent
Ox.	H IV	11.96	6,582	2.538	16,706	15,386	92.1
	J IV	12.60	6,687	2.519	16,845	15,570	92.4
	G III	13.27	6,726	2.500	16,814	15,598	92.8
	F III	13.75	6,645	2.486	16,518	15,621	94.6
	G V	13.80	7,179	2.485	17,840	17,104	95.9
	F V	13.83	6,965	2.484	17,300	17,437	100.8
	H VI	14.75	7,569	2.457	18,598	16,724	89.9
	J VI	15.46	8,032	2.437	19,573	18,375	93.9
	E III	15.68	8,776	2.430	21,325	20,742	97.3
	H V	15.73	8,668	2.429	21,054	18,535	88.0
	G III	15.81	8,171	2.426	19,822	18,234	92.0
	H IV	16.05	7,996	2.420	19,351	17,661	91.3
	F VI	16.30	7,690	2.413	18,556	17,936	96.7
	H VII	16.30	9,013	2.413	21,748	20,370	93.7
	E IV	16.41	9,909	2.409	23,871	23,557	98.7
	J V	16.59	8,877	2.404	21,341	19,293	90.4
	F IV	16.67	7,919	2.402	19,021	17,606	92.6
	J III	16.74	7,954	2.400	19,089	17,625	92.3
	E II	17.36	9,306	2.382	22,167	20,990	94.7
	D III	17.62	9,720	2.374	23,075	22,454	97.3
	D IV	17.67	10,587	2.373	25,123	24,007	95.6
	D I	18.27	8,793	2.356	20,717	19,342	93.4
	B IV	18.30	10,166	2.355	23,940	22,814	95.3
	H II	18.33	8,584	2.354	20,206	19,188	95.0
	E I	18.55	9,157	2.348	21,500	20,479	95.2
	H I	18.57	8,411	2.347	19,818	19,238	97.1
	G II	18.63	8,532	2.345	20,008	19,320	96.6
	J II	19.09	9,067	2.332	21,143	20,630	97.6
	J I	19.52	8,455	2.320	19,616	19,064	97.2
	F II	19.81	8,291	2.311	19,160	19,187	100.1
	G I	19.85	8,285	2.310	19,138	19,030	99.4
	D II	19.88	10,121	2.309	23,368	21,824	93.4
	F I	20.07	8,189	2.304	18,869	18,814	99.7
	B III	21.17	11,197	2.272	25,440	25,458	100.1
	C I	21.20	8,955	2.271	20,336	19,311	95.0
	C III	21.72	10,430	2.255	23,521	23,336	99.2
	B II	22.41	10,713	2.236	23,954	23,629	98.6
	C II	22.65	9,811	2.229	21,870	21,043	96.2
	B I	23.03	11,136	2.218	24,700	25,117	101.7



indirectly computed. Working over the data of some of Kellner's experiments³ we have computed the heat production by using equation (C), $y_2 = -0.02886x + 2.883$, and have compared with these values the heat production obtained by Kellner by indirect determination. Table II shows the results.

This group of experiments was conducted on oxen which were gaining in weight. The comparison shows that Kellner's indirectly determined heat, in all cases except four, was slightly lower than the values obtained with our equation. The range of differences is less than that in our own experiments.

The equations confirm the previous conclusion that the amounts of CO₂ and heat produced are approximately linear functions of the feed. As the feed increases in amount the CO₂ and the heat produced do not increase to the same degree; that is, their equations (A) and (B) do not represent parallel lines. As the feed increases the amount of heat produced does not increase as rapidly as the amount of CO₂ produced. In other words the ratio of CO₂ to heat has its maximum in fasting, and decreases quite regularly, but slowly, with increase in feed.

This gradual change in the relation of the amount of CO₂ and heat produced is caused by variation in the proportion of the kinds of nutriment, from the ration and from the body, which are metabolized.

Increasing amounts of feed give rise to increasing amounts of methane fermentation, with a decrease in the ratio of CO₂ to heat produced. Further, the formation of fat from carbohydrate, which takes place on heavy rations, would tend to lower this ratio. Below maintenance the oxidation of glycogen, body protein, and, finally, fat tends to increase this ratio.

The effect of the data here reported is in general to confirm, but slightly to modify, our previous determination of the mathematical relationship between the CO₂ and heat production of cattle. New equations have been deduced by means of which the heat produced can be computed without the use of a calorimeter, and more readily than by the indirect (or balance) method, in which the energy of the excreta is subtracted from the total energy of the feed, the difference being corrected for the potential energy of the gain by the body.

³Kellner, O., and Köhler, A., *Landw. Vers. Sta.*, 1900, lili, 1.

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

VI. THE ACID PROPERTIES OF REDUCED AND OXYGENATED HEMOGLOBIN.*

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The quantitative expression of the water and electrolyte distribution in the blood, and of the shifts in the distribution during respiration and other physiological changes, rests in large part on the relationship between hydron concentration and the amounts of base bound by oxygenated and reduced hemoglobin (Van Slyke, Wu, and McLean (1)). It consequently appears desirable to determine with accuracy these relationships and to construct the curves and equations expressing them. The present paper presents experimental and theoretical data directed to this end.

Christiansen, Douglas, and Haldane in 1914 (2) discovered that blood has a higher CO_2 -absorbing capacity in the reduced than in the oxygenated form, and pointed out the manner in which this property enables the blood to absorb in the tissues and expel in the lungs the observed amounts of CO_2 with the observed small changes in CO_2 tension. The results of Christiansen, Douglas, and Haldane were confirmed by Joffe and Poulton (3), by Parsons (4), and by Doisy, Briggs, Eaton, and Chambers (5).

L. J. Henderson (6) showed that the phenomena could be explained according to the law of mass action by assuming that combination of hemoglobin with a molecule of oxygen increases the acidity of a monovalent acid group in the hemoglobin molecule. Such increase in acid strength

* The results in this paper were presented at the meeting of the American Society of Biological Chemists in December, 1923, and are abstracted in the Proceedings of that meeting, this Journal (Hastings, A. B., Van Slyke, D. D., Neill, J. M., and Heidelberg, M., *J. Biol. Chem.*, 1924, lix, p. xx).

would cause the hemoglobin on oxygenation, to combine with alkali at the expense of that bound as BHCO_2 , thus freeing CO_2 for expulsion in the lungs. Conversely, deoxygenation of hemoglobin in the tissues would free alkali to combine with CO_2 , and facilitate absorption of the latter.

The effectiveness of hemoglobin as a buffer, and the interplay of the oxidation-reduction effect with the buffer effect in maintaining neutrality during the respiratory cycle have been discussed by one of the writers (Van Slyke (7, 8)).

The data available for estimation of the base-binding properties of reduced and oxygenated hemoglobin were, however, as pointed out in the introductory paper of this series (9), of such a nature as to make possible only semiquantitative approximations of the relationships involved, partly because of the limitations in accuracy of earlier methods, and partly because the experiments had been performed entirely with blood. There were no data from solutions of purified hemoglobin.

In the third paper of the present series, Van Slyke, Hastings, Heidelberger, and Neill (10) furnished experimentally for recrystallized horse hemoglobin approximate constants indicating the quantitative magnitudes of the buffer values, and of the oxidation-reduction effect on the base-binding power; and an empirical equation was developed relating the pH and the degree of oxygenation to the amount of base bound by hemoglobin in solution. The expression was

$$\begin{aligned}(1) \quad [\text{BHb}] &= \beta_o [\text{HbO}_2] [\text{pH} - a] + \beta_R [\text{Hb}_R] [\text{pH} - b] \\ &= 2.64 [\text{HbO}_2] [\text{pH} - 6.59] + 2.45 [\text{Hb}_R] [\text{pH} - 6.80]\end{aligned}$$

where $[\text{BHb}]$ was total base bound by hemoglobin, 2.64 and 2.45 were the molecular buffer values ascertained for oxygenated and reduced hemoglobin over the narrow physiological pH range, and 6.59 and 6.80 were constants near to but not assumed to be identical with the isoelectric points of oxy- and reduced hemoglobin. It was stated that the values for all four constants were to be regarded as subject to some correction due to the facts (1) that the constants 2.45 and 6.80 for reduced hemoglobin had been indirectly determined by calculation from experiments on whole blood, and (2) that correction for the amount of "inactive" hemoglobin (incapable of binding oxygen) present in the preparation was made on the unproven assumption that its buffer value and base-binding power were midway between those of reduced and oxygenated hemoglobin.

In experiments to be reported in this paper we have determined directly the base-binding power and buffer value of both oxygenated hemoglobin and reduced hemoglobin over the pH range 6.8 to 7.6. Similar data on inactive hemoglobin have been obtained, and have been found to coincide with those for reduced hemoglobin¹ so that it becomes possible to correct accurately for the effect of the inactive hemoglobin in the oxygenated and reduced solu-

tions. With the more accurate data thus obtained, theoretical deductions have been reached concerning the chemical changes in hemoglobin which result from oxygenation and reduction, and which cause the variation in base-binding power that accompanies variation in degree of oxygen saturation.

Methods.

The hemoglobin used in most of the experiments was prepared as described by Heidelberger (11). In Experiment 6 an attempt was made to purify hemoglobin more completely by introducing a preliminary salting out of the cell globulins. Washed red cells were laked with 5 volumes of ice water saturated with ether, which had been purified by washing with water followed by drying first over calcium chloride and finally over solid sodium hydroxide. Ether purified in this way does not convert oxyhemoglobin to methemoglobin. After having stood overnight the solution was treated with an equal volume of saturated ammonium sulfate solution, and was then siphoned off as rapidly as possible through a number of large folded filters to remove the globulin precipitate. The filtrate was allowed to stand in the refrigerator overnight for the oxyhemoglobin to crystallize. Crystallization often began before the filtration was complete, and the substance separated in particularly fine, large, glistening crystals. The supernatant liquor was decanted and the crystals were collected on a large Büchner funnel and washed with small portions of water saturated with 4:1 carbon dioxide-oxygen mixture. Recrystallization was then carried out as in Heidelberger's method. Since the results obtained in this experiment were identical with those obtained from hemoglobin prepared throughout by Heidelberger's original method, the latter was used for the preparations employed in the other experiments.

Solutions were made of such strength that the concentration of hemoglobin was approximately 7.0 mm. The alkali used for solution of the hemoglobin was at various times 30 mm NaOH, 30 mm KOH, 50 mm KOH, and 30 mm KOH plus 115 mm KCl. The 50 mm KOH was used in two experiments in order to obtain pH values above 7.6 without lowering p_{CO_2} so far as to prevent accurate estimation of the $\frac{\text{H}_2\text{CO}_3}{\text{HCO}_3}$ ratio.

In determining the CO_2 absorption curves, the technique used was that already described by Van Slyke, Hastings, Heidelberger, and Neill (10) with the simplification introduced by Van Slyke, Wu, and McLean, whereby the initial gas tensions are approximated by pressure rather than volume measurements. With each hemoglobin solution one CO_2 absorption curve was determined on an oxygenated portion, and one curve on a portion reduced with hydrogen. The temperature was always 38° .

In the earlier experiments of those here presented the concentrations of total base were estimated from the amounts of alkali added to the hemoglobin solutions, the hemoglobin preparations being assumed to be base-free. In the later experiments the total base concentration was directly determined by a slight modification of the method published by Van Slyke, Wu, and McLean (1), which adapted it for small amounts of material. With this method a series of experiments was run to determine the efficiency of the method of dialysis used by Heidelberger (11). These experiments were carried out as follows:

Experiment to Determine the Rate at Which Base Is Dialyzed from Hemoglobin through Collodion Bags.

Days dialyzed.	Conductivity of saturated solution $\times 10^4$.	Total Hb in saturated solution.	Determinations on Hb samples taken from collodion bags.					Remarks.
			Total base.	Total base in control.	Net base in Hb.	Total Hb.	Total base Total Hb	
	mhos.	mm per l.	m.-Eq. per l.	m.-Eq. per l.	m.-Eq. per l.	mm per l.	mm	
0	12.4	0.96	25.4	3.8	21.6	13.2	1.64	Analyses on mixed samples of Hb crystals and supernatant solution taken from bags.
2	2.7	0.58	17.2	3.8	13.4	9.6	1.40	
3	4.7	0.74	12.5	3.0	9.5	10.2	0.94	
5	2.3	0.68	12.5	2.4	10.1	10.6	0.95	
7			7.8	6.5	1.3	14.2	0.09	Analyses on washed crystals.
7	17.2	2.28	22.8				10.0	Analyses on supernatant solution.

Determinations of the conductivity and hemoglobin content of saturated solutions of the hemoglobin crystals were made on successive days after dialysis was begun. These results are shown in Columns 2 and 3 of the above table. At the same time determinations were made of the total base and hemoglobin content of mixed samples of the hemoglobin crystals taken from the collodion bags. The millimols of total base per millimol of hemoglobin are given in Column 8. The results of these analyses indicate that a considerable amount of base is left in the collodion sacs even after 6 days dialysis. That this

base is not in the crystals, but in the mother liquor, however, is shown by the fact that upon filtering and washing the crystalline hemoglobin a negligible amount of base was found in it, whereas the supernatant solution contained a considerable amount. The data therefore support the assumption that the washed crystals can be obtained practically free from base.

Calculations and Symbols.

The pH has been calculated from Hasselbalch's formula

$$\text{pH} = \text{pK}' + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$$

pK' was found by simultaneous H_2CO_3 , BHCO_3 , and electro-metric pH determinations to be 6.17 for 7 mm hemoglobin solutions in which the cation concentration was either 30 or 50 mm and 6.11 for solutions whose cation concentration was 145 mm, $[\text{H}_2\text{CO}_3]$ being estimated by means of the α_{CO_2} determined as given below.

$$[\text{BHCO}_3] = [\text{CO}_2] - [\text{H}_2\text{CO}_3]$$

$$[\text{H}_2\text{CO}_3] = \frac{\alpha_{\text{CO}_2} \times p_{\text{CO}_2}}{760 \times 0.0224} = 0.0587 \alpha_{\text{CO}_2} p_{\text{CO}_2}$$

p_{CO_2} = tension of CO_2 in mm. of Hg.

$[\text{CO}_2]$ = total concentration of CO_2 in mm per liter of solution.

α_{CO_2} = $0.555 \times \text{H}_2\text{O}$.

H_2O = kg. of H_2O in 1 liter of solution.

The calculation of α_{CO_2} for our hemoglobin solutions as $0.555 \times \text{H}_2\text{O}$ was adopted as the result of experiments in which the solubility coefficient of CO_2 in acidified hemoglobin solutions at 38°C . was determined. These solubility coefficients were in accordance with the above empirical formula.

The following symbols are used for hemoglobin and its salts.

Hb = total hemoglobin, expressed in mols of oxygen capacity.

BHb = equivalents of base bound by total hemoglobin.

BHbO_2 = " " " " oxyhemoglobin.

BHb_2 = " " " " reduced hemoglobin.

$\frac{d[\text{BHb}]}{d[\text{O}_2\text{Hb}]}$ or $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ = increase in base bound by hemoglobin with increase in oxygen bound by hemoglobin at constant pH and hemoglobin concentration.

The expression $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ suggests its significance, increase in B bound by Hb, somewhat better than does increase in O₂ bound by Hb, $\frac{\Delta[\text{BHb}]}{\Delta[\text{HbO}_2]}$. For this reason we have reversed in this expression the usual HbO₂ symbol.

The base bound by the hemoglobin has been calculated from the formula

$[\text{BHb}] = [\text{B}] - [\text{BHCO}_2]$ where

$[\text{BHb}]$ = base bound by total reduced, oxygenated, and inactive hemoglobin = $[\text{BHb}_\text{R}] + [\text{BHbO}_2]$

$[\text{B}]$ = total base present (except that added as chloride) = $[\text{BHb}] + [\text{BHCO}_2]$

All the concentrations $[\text{Hb}]$, $[\text{BHCO}_2]$, $[\text{H}_2\text{CO}_3]$, etc., in the present paper are given in terms of the volume ratio $\frac{\text{millimols solute}}{\text{liter solution}}$.

Since no osmotic phenomena are considered, it is not necessary to use the $\frac{\text{solute}}{\text{water}}$ ratio employed by Van Slyke, Wu, and McLean (1). The volume ratio is consequently used here for its greater convenience.

As in Paper V (1), $[\text{Hb}]$ is used to indicate the total hemoglobin concentration in place of the less distinctive C employed in Papers III and IV. The other symbols are consistent with those of the previous papers in this series.

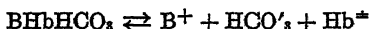
We have used, as in Paper III (9), the symbols β_0 and β_R for molecular buffer values of oxygenated and reduced hemoglobin.

$$\beta_0 = \frac{d [\text{BHbO}_2]}{[\text{HbO}_2] \text{ dpH}}$$

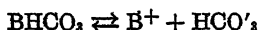
$$\beta_\text{R} = \frac{d [\text{BHb}_\text{R}]}{[\text{Hb}_\text{R}] \text{ dpH}}$$

The base calculated as BHb by the above equation, $[\text{BHb}] = [\text{B}] - [\text{BHCO}_2]$, in reality represents *excess of base* (Na or K) *over acid* (H_2CO_3 or HCl) *bound by hemoglobin*. Some of the $[\text{BHCO}_2]$ estimated as $[\text{CO}_2] - [\text{H}_2\text{CO}_3]$, even on the alkaline side of the isoelectric point of hemoglobin, is presumably bound

to the protein, the B by the acid groups, the HCO_3 by the amino groups, the result being a double salt most simply represented as $\text{BHb}(\text{HCO}_3)$. If such protein double salts are ionized to the same extent as BHCO_3 , however, the ionic concentrations will be the same, whether formed by the reaction



or by the reaction



Absolutely complete reduction of hemoglobin with hydrogen is difficult to attain, and in most cases was only approximated in our experiments. The oxygen contents of the reduced solutions were determined, however, and the $[\text{BHb}]$ corresponding to complete reduction was estimated by extrapolation according to the fact previously established (9) that the $\frac{d[\text{BHb}]}{d[\text{O}_2\text{Hb}]}$ ratio at a given pH is constant, and unaffected by the degree of oxygenation.

Complete oxygenation is likewise difficult to obtain in solutions of purified hemoglobin, because a few per cent of the hemoglobin is usually inactivated (changed to methemoglobin) in the process of preparation. It will be shown in a separate paper that the inactive hemoglobin binds the same amounts of base as reduced hemoglobin. On this basis the observed $[\text{BHb}]$ values are extrapolated, not only for complete reduction, as described above, but also for complete oxygenation. The data thus estimated are given in parentheses in Tables I a, II a. . . . XV a.

RESULTS.

The results of our experiments with thirteen preparations of recrystallized horse hemoglobin and two of dog hemoglobin are given in Tables I to XV and Figs. 1 to 15. As indicated in the summarizing Tables XVI and XVIII, sodium salts were used in some experiments, potassium in others, the results being, within the limit of experimental error, identical with both.

TABLE I.

Experiment on Horse Hemoglobin No. H 16. Oct. 17, 1922.

Total H_2O = 870.0 gm. per liter.
 " Hb = 9.22 mM " "
 " HbO_2 = 8.44 " " "
 " NaOH = 30.78 " " "
 Conductivity = 8.6×10^{-5}
 $\alpha_{\text{CO}_2} = 0.555 \times 0.870 = 0.4828$
 $\text{H}_2\text{CO}_3 = p_{\text{CO}_2} \times 0.02834$
 $\text{pK}' = 6.17$

No.	PO_2	Total $[\text{O}_2]$	$[\text{HbO}_2]$	PCO_2	$[\text{H}_2\text{CO}_3]$	Total $[\text{CO}_2]$	$[\text{BHCO}_2]$	pH	$[\text{BHb}]$
	mm.	mM	mM	mm.	mM	mM	mM		m.-Eq.
1	(135)	8.06	7.90	55.7	1.579	18.71	17.13	7.205	13.65
2	(135)	8.06	7.90	40.4	1.145	16.15	17.01	7.202	13.77
3	139.6	8.22	8.06	27.8	0.7880	13.30	12.51	7.370	18.27
4	139.2	8.08	7.92	19.3	0.547	10.91	10.36	7.447	20.42
5	4.0	0.53	0.53	69.4	1.967	23.47	21.50	7.209	9.28
6	4.8	0.66	0.66	52.2	1.480	20.94	19.46	7.289	11.32
7	0.0	0.74	0.74	36.6	1.038	18.13	17.09	7.386	13.69
8	0.0	1.08	1.08	25.0	0.7085	15.23	14.52	7.482	16.26

TABLE I a.

*Summary of Results on Horse Hemoglobin No. H 16. Oct. 17, 1922.*Total $[\text{Hb}] = 9.22$ mM per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$
	$[\text{HbO}_2]$	$[\text{BHb}]$	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	$[\text{HbO}_2]$	$[\text{BHb}]$	$\frac{[\text{BHb}_R]}{[\text{Hb}_R]}$	
	mM	m.-Eq.	m.-Eq.	mM	m.-Eq.	m.-Eq.	m.-Eq.
7.2	7.90 (9.22)*	13.44 (14.23)	(1.54)	0.53 (0.00)	9.0 (8.68)	(0.94)	0.602
7.4	7.92 (9.22)	18.94 (19.83)	(2.15)	0.80 (0.00)	14.05 (13.50)	(1.46)	0.687

pH 7.3
 β_0 3.05
 β_z 2.6

* The numbers enclosed in parentheses indicate values for complete oxygenation or reduction extrapolated from the experimental values directly above them.

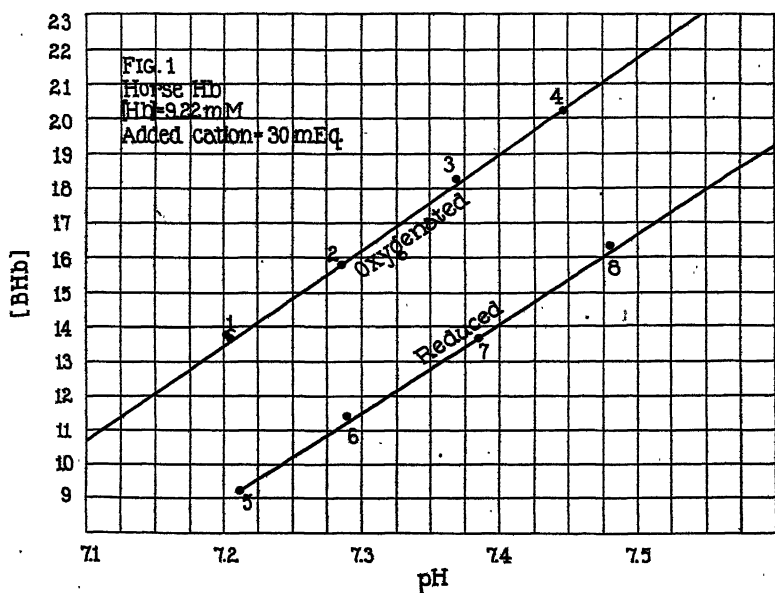


FIG. 1.

Total H₂O = 891.0 gm. per liter.

" Hb = 7.73 mm " "

" HbO₂ = 6.92 " " "

" NaOH = 30.78 " " "

Conductivity = 4.4×10^{-5}

$\alpha_{\text{CO}_2} = 0.555 \times 0.891 = 0.4943$

H₂CO₃ = p_{CO₂} \times 0.02904

pK' = 6.17

No.	P _{O₂}	Total [O ₂]	[HbO ₂]	P _{CO₂}	[H ₂ CO ₃]	Total [CO ₂]	[BHCO ₃]	pH	[BHb]
	mm.	mm	mm	mm.	mm	mm	mm		m.-Eq.
1	(135)	6.63	6.46	62.0	1.800	21.23	19.43	7.203	11.35
		6.63		62.0	1.800	20.75	18.95	7.192	11.83
2	(135)	6.69	6.52	43.3	1.257	17.87	16.61	7.291	14.17
						17.94	16.68	7.291	14.10
3	(135)	6.68	6.51	30.1	0.874	15.31	14.44	7.388	16.34
4	(135)	6.68	6.51	21.6	0.627	13.20	12.57	7.472	18.21
5	(0)	0.36	0.36	72.4	2.103	24.70	22.60	7.202	8.18
6	(0)	0.35	0.35	54.7	1.588	22.34	20.75	7.286	10.03
7	(0)	0.40	0.40	38.8	1.127	19.93	18.80	7.392	11.98
8	(0)	0.73	0.73	27.7	0.804	17.29	16.49	7.482	14.27
9	(135)	5.6	5.04	504.0	14.63	49.50	34.87	6.547	-4.09
						49.30	34.67	6.545	-3.89
10	(0)	0.22	0.22	307.5	8.93	41.9	32.97	6.737	-2.19
						42.0	33.07	6.739	-2.29

TABLE II a.

Summary of Results on Horse Hemoglobin No. H 18. Oct. 19,
Total [Hb] = 7.73 mm per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$
	[HbO ₂]	[BHb]	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	[HbO ₂]	[BHb]	$\frac{[\text{BHb}_R]}{[\text{Hb}_R]}$	
	mm	m.-Eq.	m.-Eq.	mm	m.-Eq.	m.-Eq.	m.-Eq.
6.8	6.15 (7.73)*	2.1 (2.90)	(0.38)	0.22 (0.00)	-0.9 (-1.01)	(-0.13)	0.506
7.0	6.35 (7.73)	6.9 (7.67)	(0.99)	0.27 (0.00)	3.5 (3.35)	(0.43)	0.559
7.2	6.45 (7.73)	11.7 (12.48)	(1.61)	0.32 (0.00)	7.95 (7.75)	(1.00)	0.612
7.4	6.51 (7.73)	16.5 (17.32)	(2.24)	0.40 (0.00)	12.4 (12.13)	(1.57)	0.671

pH	6.9	7.1	7.3
β_o	3.05	3.1	3.15
β_R	2.8	2.85	2.85

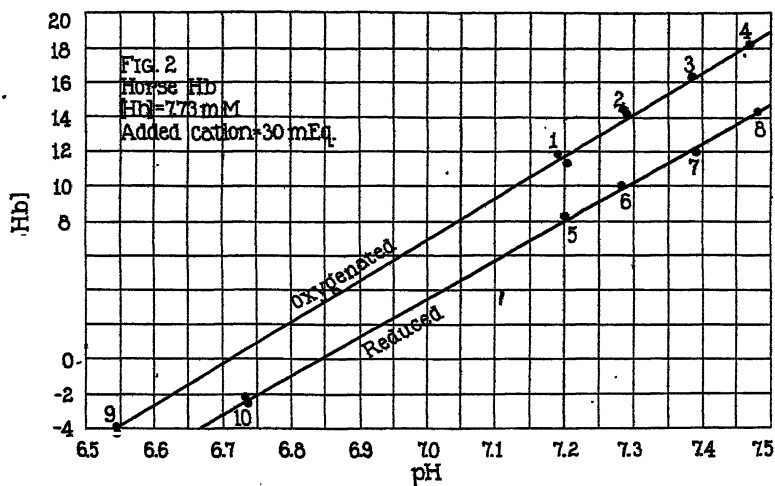


FIG. 2.

" Hb = 7.48 mm " "
 " HbO₂ = 7.04 " " "
 " NaOH = 30.78 " " "
 Conductivity 3.5×10^{-5}
 $\alpha_{\text{CO}_2} = 0.555 \times 0.895 = 0.497$
 $\text{H}_2\text{CO}_3 = p_{\text{CO}_2} \times 0.0292$
 $\text{pK}' = 6.17$

No.	P _{O2}	Total [O ₂]	[HbO ₂]	P _{CO2}	[H ₂ CO ₃]	Total [CO ₂]	[BHCO ₂]	pH	[BHb]
	mm.	mm	mm	mm.	mm	mm	mm		m.-Eq.
1	(135)	6.50	6.34	195.3	32.91	5.70	27.21	6.849	3.57
2	(0)	0.21	0.21	232.3	37.30	6.78	30.52	6.824	0.26
3	(135)	6.53	6.37	106.3	25.90	3.104	22.80	7.036	7.98
4	(0)	0.25	0.25	124.5	29.81	3.634	26.18	7.028	4.60
5	(135)	6.68	6.52	56.3	20.25	1.643	18.61	7.224	12.17
6	(0)	0.44	0.44	69.2	24.47	2.020	22.45	7.216	8.33
7	(135)	6.68	6.52	28.1	15.32	0.820	14.50	7.417	16.28
8	(0)	0.51	0.51	37.2	19.74	1.086	18.65	7.405	12.13
9	(135)	6.74	6.58	13.6	11.16	0.397	10.76	7.603	20.02
10	(0)	0.49	0.49	18.9	15.75	0.552	15.20	7.610	15.58

TABLE III a.

Summary of Results on Horse Hemoglobin No. H 24. Nov. 29, 1922.

Total [Hb] = 7.48 mm per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$
	[HbO ₂]	[BHb]	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	[HbO ₂]	[BHb]	$\frac{[\text{BHb}_2]}{[\text{Hb}_2]}$	
	mm	m.-Eq.	m.-Eq.	mm	m.-Eq.	m.-Eq.	m.-Eq.
6.8	6.30 (7.48)*	2.40 (2.87)	(0.38)	0.21 (0.00)	0.00 (-0.08)	(-0.01)	0.394
7.0	6.35 (7.48)	7.10 (7.66)	(1.02)	0.25 (0.00)	4.10 (3.98)	(0.53)	0.492
7.2	6.50 (7.48)	11.60 (12.16)	(1.63)	0.40 (0.00)	8.08 (7.85)	(1.05)	0.577
7.4	6.52 (7.48)	16.0 (16.64)	(2.22)	0.51 (0.00)	12.05 (11.71)	(1.56)	0.657
7.6	6.57 (7.38)	20.10 (20.80)	(2.78)	0.49 (0.00)	15.40 (15.02)	(2.01)	0.773

pH	6.9	7.1	7.3	7.5
β_0	3.2	3.05	2.95	2.8
β_2	2.7	2.6	2.6	2.25

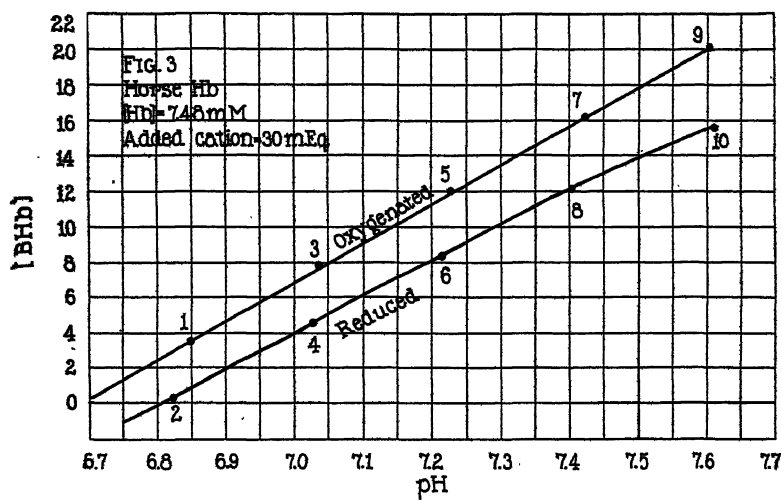


FIG. 3.

Total H_2O = 894.0 gm. per liter.

" Hb = 7.48 mm " "

" HbO_2 = 6.90 " " "

" NaOH = 30.78 " " "

Conductivity = 4.7×10^{-5}

$\alpha_{\text{CO}_2} = 0.555 \times 0.894 = 0.496$

$\text{H}_2\text{CO}_3 = p_{\text{CO}_2} \times 0.02914$

$pK' = 6.17$

No.	p_{O_2}	Total $[\text{O}_2]$	$[\text{HbO}_2]$	p_{CO_2}	$[\text{H}_2\text{CO}_3]$	Total $[\text{CO}_2]$	$[\text{BHCO}_3]$	pH	$[\text{BHb}]$
	mm.	mm	mm	mm.	mm	mm	mm		m.-Eq.
1	(135)	6.26	6.10	200.2	5.840	34.15	28.31	6.857	2.47
2	(0)	0.24	0.24	230.2	6.705	38.17	31.46	6.841	-0.68
3	(135)	6.46	6.30	107.8	3.142	26.84	23.70	7.047	7.08
4	1.85	0.20	0.20	127.6	3.720	31.00	27.28	7.035	3.50
5	(135)	6.55	6.39	57.1	1.665	20.98	19.31	7.234	11.47
6	1.70	0.26	0.26	71.4	2.082	25.66	23.58	7.224	7.20
7	(135)	6.51	6.35	28.8	0.840	16.07	15.23	7.428	15.55
8	1.67	0.50	0.50	38.8	1.131	20.49	19.36	7.404	11.42
9	(135)	6.47	6.31	13.5	0.394	11.82	11.43	7.633	19.35
10	2.34	0.59	0.59	18.7	0.545	16.10	15.55	7.625	15.23

TABLE IV a.

Summary of Results on Horse Hemoglobin No. H 25. Dec. 5,

Total $[\text{Hb}] = 7.48$ mm per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$
	$[\text{HbO}_2]$	$[\text{BHb}]$	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	$[\text{HbO}_2]$	$[\text{BHb}]$	$\frac{[\text{BHb}_R]}{[\text{Hb}_R]}$	
	mm	m.-Eq.	m.-Eq.	mm	m.-Eq.	m.-Eq.	m.-Eq.
6.8	6.05 (7.48)*	1.10 (1.73)	(0.23)	0.23 (0.00)	-1.05 (-1.55)	(-0.21)	0.438
7.0	6.25 (7.48)	5.95 (6.59)	(0.88)	0.20 (0.00)	2.8 (2.70)	(0.36)	0.521
7.2	6.37 (7.48)	10.73 (11.41)	(1.53)	0.26 (0.00)	6.98 (6.82)	(0.91)	0.614
7.4	6.36 (7.48)	15.0 (15.71)	(2.10)	0.50 (0.00)	11.30 (10.98)	(1.47)	0.632
7.6	6.30 (7.48)	18.77 (19.55)	(2.61)	0.57 (0.00)	15.0 (14.62)	(1.95)	0.658

pH	6.9	7.1	7.3	7.5
β_0	3.25	3.25	2.85	2.85
β_R	2.85	2.75	2.80	2.4

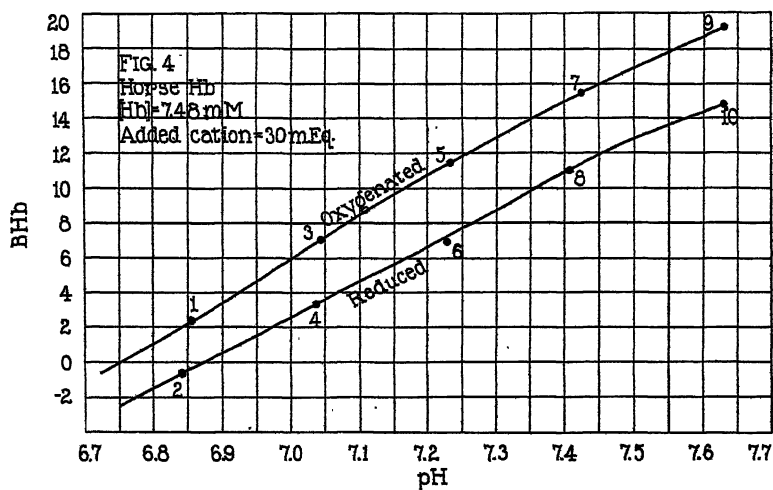


FIG. 4.

TABLE V.

*Experiment on Horse Hemoglobin No. H 26. Dec. 7, 1922.*Total H_2O = 880.0 gm. per liter.

" Hb = 8.48 mm " "

" HbO_2 = 8.38 " " "

" NaOH = 30.78 " " "

Conductivity = 4.5×10^{-5} $\alpha_{\text{CO}_2} = 0.555 \times 0.880 = 0.4883$ $\text{H}_2\text{CO}_3 = p_{\text{CO}_2} \times 0.02870$ $\text{pK}' = 6.17$

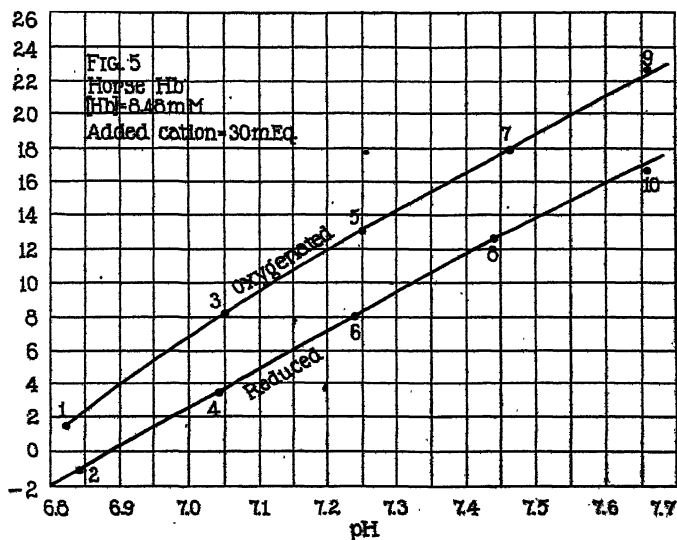
No.	P_{O_2}	Total [O_2]	[Hb O_2]	P_{CO_2}	[H_2CO_3]	Total [CO_2]	[BH CO_2]	pH	[BHb]
	mm.	mm	mm	mm.	mm	mm	mm		m.-Eq.
1	(135)	7.43	7.27	227.4	6.530	35.81	29.28	6.821	1.50
2	(0)	0.23	0.23	234.5	6.732	38.53	31.80	6.844	-1.02
3	(135)	7.64	7.48	103.5	2.970	25.62	22.65	7.052	18.13
4	2.9	0.25	0.25	127.0	3.646	30.87	27.22	7.043	3.56
5	(135)	7.80	7.64	50.9	1.461	19.16	17.70	7.254	13.08
6	3.0	0.34	0.34	67.7	1.943	24.74	22.80	7.239	7.98
7	(135)	7.91	7.75	22.8	0.6543	13.53	12.88	7.464	17.90
8	2.5	0.62	0.62	33.8	0.971	19.10	18.13	7.441	12.65
9	(135)	7.98	7.72	9.2	0.264	6.51	8.25	7.665	22.53
10	(0)	0.78	0.78	15.8	0.4535	14.44	13.99	7.659	16.79
11	(135)	7.87	7.71	2.25	0.646	3.75	3.10	7.851	27.68
12	(0)	1.77	1.77	6.58	0.1889	9.13	8.94	7.845	21.84

Total [Hb] = 8.48 mm per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta [\text{BHb}]}{\Delta [\text{O}_2\text{Hb}]}$
	[HbO ₂]	[BHb]	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	[HbO ₂]	[BHb]	$\frac{[\text{BHb}_2]}{[\text{Hb}_2]}$	
	mm	m.-Eq.	m.-Eq.	mm	m.-Eq.	m.-Eq.	m.-Eq.
6.8	7.27 (8.48)*	1.0 (1.52)	(0.18)	0.22 (0.00)	-2.0 (-2.9)	(-0.34)	0.426
7.0	7.42 (8.48)	6.8 (7.44)	(0.88)	0.25 (0.00)	2.5 (2.35)	(0.28)	0.600
7.2	7.57 (8.48)	12.0 (12.62)	(1.49)	0.33 (0.00)	7.03 (6.80)	(0.80)	0.686
7.4	7.73 (8.48)	16.58 (17.10)	(2.02)	0.56 (0.00)	11.62 (11.23)	(1.32)	0.692
7.6	7.75	21.03 (21.56)	(2.55)	0.72 (0.00)	15.92 (15.40)	(1.82)	0.727

pH	6.9	7.1	7.3	7.5
β_o	3.5	3.05	2.65	2.65
β_R	3.10	2.60	2.60	2.50

* The numbers enclosed in parentheses indicate values for complete oxygenation or reduction extrapolated from the experimental values directly above them.



Experiment on Horse Hemoglobin No. H 32. Dec. 27,

Total H₂O = 920.0 gm. per liter.

" Hb = 5.69 mm " "

" HbO₂ = 5.03 " " "

" NaOH = 30.0 " " "

$$\alpha_{\text{CO}_2} = 0.555 \times 0.920 = 0.5108$$

$$\text{H}_2\text{CO}_3 = p_{\text{CO}_2} \times 0.03$$

$$pK' = 6.17$$

No.	P _{O₂}	Total [O ₂]	[HbO ₂]	P _{CO₂}	[H ₂ CO ₃]	Total [CO ₂]	[BHCO ₃]	pH	[BHb]
	mm.	mm	mm	mm.	mm	mm	mm		m.-Eq.
1	(135)	4.49	4.33	209.4	6.282	34.05	27.77	6.815	2.23
2	(0)	0.13	0.13	230.9	6.927	36.70	29.77	6.803	0.23
3	(135)	4.53	4.37	118.3	3.549	27.69	24.14	7.003	5.86
4	(0)	0.13	0.13	133.9	4.017	30.76	26.74	6.994	3.26
5	(135)	4.62	4.46	68.0	2.040	23.31	21.27	7.188	8.73
6	(0)	0.32	0.32	77.9	2.337	26.12	23.78	7.177	6.22
7	(135)	4.71	4.55	38.1	1.143	19.28	18.14	7.370	11.86
8	(0)	0.20	0.20	44.7	1.341	22.56	21.22	7.369	8.78
9	(135)	4.65	4.49	22.9	0.687	16.42	15.73	7.530	14.27
10	(0)	0.39	0.39	25.2	0.756	19.03	18.27	7.553	11.73

TABLE VI a.

Summary of Results on Horse Hemoglobin No. H 32. Dec. 27, 1922.

Total [HB] = 5.69 mm per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$
	[HbO ₂]	[BHb]	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	[HbO ₂]	[BHb]	$\frac{[\text{BHb}_2]}{[\text{Hb}_2]}$	
	mm	m.-Eq.	m.-Eq.	mm	m.-Eq.	m.-Eq.	m.-Eq.
6.8	4.33 (5.69)*	1.90 (2.35)	(0.41)	0.13 (0.00)	0.23 (0.29)	(0.03)	0.398
7.0	4.37 (5.69)	5.85 (6.57)	(1.15)	0.13 (0.00)	3.40 (3.33)	(0.59)	0.573
7.2	4.46 (5.69)	9.02 (9.79)	(1.72)	0.32 (0.00)	6.35 (6.15)	(1.08)	0.621
7.4	4.55 (5.69)	12.24 (12.98)	(2.28)	0.20 (0.00)	9.40 (9.27)	(1.63)	0.653

pH	6.9	7.1	7.3
β_0	3.7	2.85	2.8
β_2	2.85	2.5	2.75

* The numbers enclosed in parentheses indicate values for complete oxygenation or reduction extrapolated from the experimental values

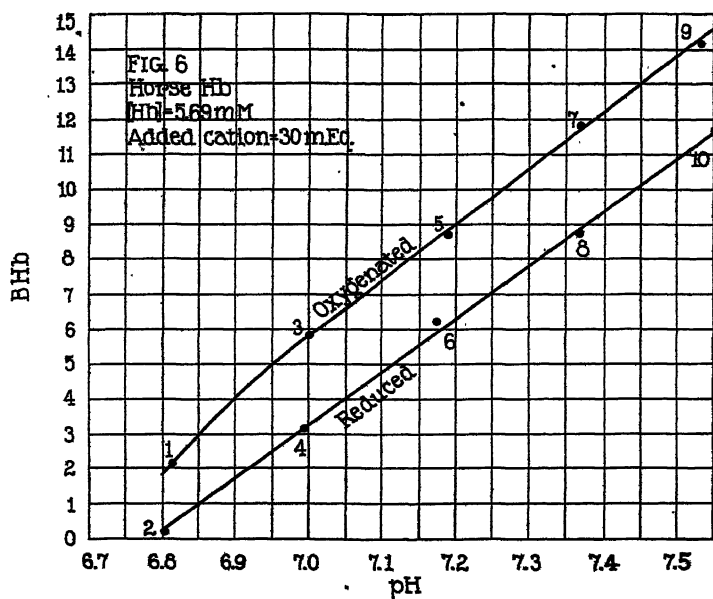


FIG. 6.

Experiment on Horse Hemoglobin No. H 43. Mar. 16, 1923.

Total H_2O = 885.0 gm. per liter.

" Hb = 8.87 mm " "

" HbO_2 = 7.82 " " "

" KOH = 26.88 " " "

$$\alpha_{CO_2} = 0.555 \times 0.885 = 0.491$$

$$H_2CO_3 = p_{CO_2} \times 0.02886$$

$$pK' = 6.17$$

No.	PO_2	Total $[O_2]$	$[HbO_2]$	p_{CO_2}	$[H_2CO_3]$	Total $[CO_2]$	$[BHCO_3]$	pH	$[BHb]$
	mm.	mm	mm	mm.	mm	mm	mm		m.-Eq.
1	(135)	5.60	5.44	211.1	6.095	31.03	24.93	6.782	1.95
2	(0)	0.44	0.44	226.8	6.545	33.94	27.39	6.792	-0.51
3	(135)	7.21	7.05	112.3	3.241	23.07	19.83	6.956	7.05
4	(0)	0.22	0.22	132.6	3.826	27.10	23.27	6.954	3.61
5	(135)	7.40	7.24	60.7	1.753	17.45	15.70	7.122	11.18
6	(0)	0.18	0.18	68.8	1.986	21.87	19.88	7.170	7.00
7	(135)	7.22	7.06	32.2	0.9295	12.89	11.96	7.280	14.92
8	(0)	0.37	0.37	40.9	1.181	16.98	15.80	7.296	11.08
9	(135)	7.30	7.14	15.1	0.4357	8.79	8.35	7.452	18.53
10	(0)	0.99	0.99	20.8	0.600	12.44	11.84	7.465	15.04

TABLE VII a.

Summary of Results on Horse Hemoglobin No. H 43. Mar. 16, 1923.

Total $[Hb]$ = 8.87 mm per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta [BHb]}{\Delta [O_2Hb]}$
	$[HbO_2]$	$[BHb]$	$\frac{[BHbO_2]}{[HbO_2]}$	$[HbO_2]$	$[BHb]$	$\frac{[BHb_B]}{[Hb_B]}$	
	mm	m.-Eq.	m.-Eq.	mm	m.-Eq.	m.-Eq.	m.-Eq.
6.8	5.44 (8.87)*	2.55 (3.78)	(0.43)	0.32	-0.3 (-0.46)	(-0.05)	0.504
7.0	7.10 (8.87)	8.2 (9.22)	(1.04)	0.20	4.23 (4.11)	(0.46)	0.575
7.2	7.15 (8.87)	13.03 (14.08)	(1.59)	0.22	8.8 (8.66)	(0.98)	0.610
7.4	7.13 (8.87)	17.43 (18.50)	(2.09)	0.73	13.40 (12.94)	(1.46)	0.630

pH	6.9	7.1	7.3
β_o	3.05	2.75	2.5
β_H	2.55	2.60	2.4

The numbers enclosed in parentheses indicate values for complete oxygenation or reduction extrapolated from the experimental values

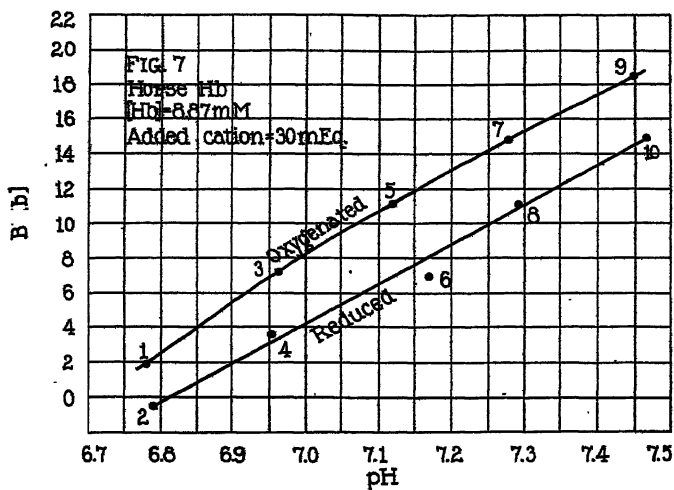


FIG. 7.

TABLE VIII.

Experiment on Horse Hemoglobin No. H 45. Mar. 27, 1923.

Total H₂O = 919.7 gm. per liter.

" Hb = 5.70 mm " "

" HbO₂ = 4.47 " " "

" KOH = 30.0 " " "

 $\alpha_{\text{CO}_2} = 0.555 \times 0.9197 = 0.510$ $\text{H}_2\text{CO}_3 = p_{\text{CO}_2} \times 0.03$ $pK' = 6.17$

No	P _{O₂}	Total [O ₂]	[HbO ₂]	P _{CO₂}	[H ₂ CO ₃]	Total [CO ₂]	[BHCO ₂]	pH	[BHb]
	mm.	mm	mm	mm.	mm	mm	mm		m.-Eq.
1	(0)	0.24	0.24	231.1	6.933	37.06	30.13	6.808	-0.13
2	(135)	4.19	4.03	201.4	6.042	33.66	27.62	6.830	2.88
3	(0)	0.38	0.38	136.4	4.092	31.15	27.06	6.901	2.94
4	(135)	4.23	4.07	124.8	3.744	28.82	25.08	6.996	4.92
5	(0)	0.31	0.31	77.2	2.316	26.61	24.29	7.190	5.71
6	(135)	4.21	4.05	63.5	1.905	23.49	21.58	7.224	8.42
7	(0)	0.36	0.36	43.5	1.305	22.68	21.37	7.384	8.63
8	(135)	4.26	4.10	48.1	1.443	21.33	19.89	7.309	10.11

TABLE VIII a.

Summary of Results on Horse Hemoglobin No. H 45. Mar. 27,

Total [Hb] = 5.70 mm per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta [\text{BHb}]}{\Delta [\text{O}_2\text{Hb}]}$
	[HbO ₂]	[BHb]	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	[HbO ₂]	[BHb]	$\frac{[\text{BHb}_2]}{[\text{Hb}_2]}$	
	mm	m.-Eq.	m.-Eq.	mm	m.-Eq.	m.-Eq.	m.-Eq.
6.8	4.02 (5.70)*	1.9 (2.7)	(0.47)	0.25 (0.00)	0.1 (-0.02)	(-0.004)	0.477
7.0	4.07 (5.70)	4.97 (5.84)	(1.03)	0.38 (0.00)	3.0 (2.8)	(0.49)	0.534
7.2	4.05 (5.70)	8.05 (9.00)	(1.58)	0.30 (0.00)	5.9 (5.73)	(1.01)	0.573
7.4	4.10 (5.70)	11.15 (12.15)	(2.13)	0.35 (0.00)	8.8 (8.58)	0.627 (1.51)	0.627

pH	6.9	7.1	7.3
β_{O}	3.7	2.75	2.8
β_{H}	2.6	2.5	2.65

*The numbers enclosed in parentheses indicate values for complete oxygenation or reduction extrapolated from the experimental values directly above them.

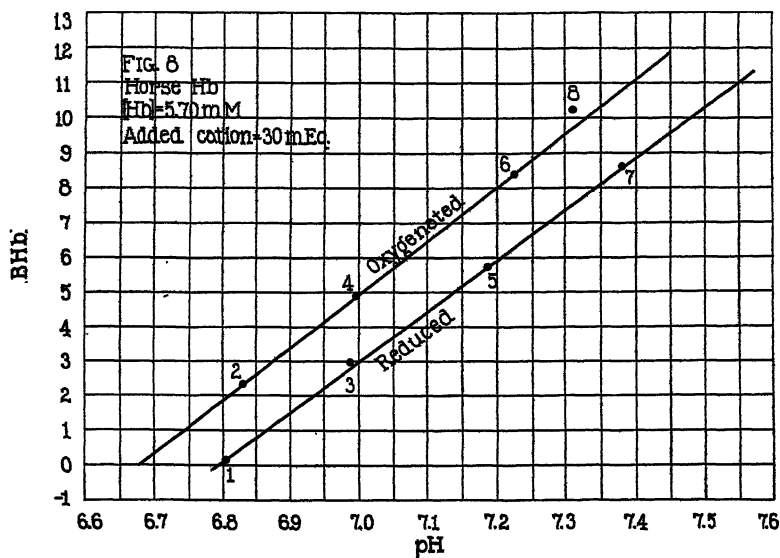


FIG. 8.

TABLE IX.

*Experiment on Horse Hemoglobin No. H 58. June 14, 1923.*Total H_2O = 902.0 gm. per liter.

" Hb = 7.08 mm " "

" HbO_2 = 6.24 " " "

" KOH = 30.0 " " "

 $\alpha_{CO_2} = 0.555 \times 0.902 = 0.501$ $H_2CO_3 = p_{CO_2} \times 0.0294$ $pK' = 6.17$

No.	P_{O_2}	Total [O_2].	[Hb O_2]	P_{CO_2}	[H_2CO_3]	Total [CO_2].	[BH CO_2]	pH	[BHb]
	mm.	mm	mm	mm.	mm	mm	mm		m.-Eq.
1	(135)	5.78	5.62	201.8 200.8	5.935 5.902	33.04	27.10 27.14	6.830 6.833	2.90 2.86
2	(0)	0.05	0.05	230.3	6.770	36.48	29.71	6.813	0.29
3	(135)	5.89	5.73	107.0	3.146	25.83	22.68	7.028	7.32
4	(0)	0.04	0.04	129.4	3.804	30.11	26.31	7.010	3.60
5	(135)	6.00	5.84	56.6	1.664	20.25	18.59	7.218	11.41
6	(0)	0.08	0.08	68.9	2.026	24.35	22.32	7.212	7.68
7	(135)	6.07	5.91	28.6	0.841	15.59	14.75	7.414	15.25
8	(0)	0.17	0.17	38.4	1.129	20.03	18.90	7.394	11.10
9	(135)	6.11	5.95	13.4	0.394	11.12	10.73	7.605	19.27
10	(0)	0.38	0.38	19.8	0.582	15.94	15.36	7.591	14.64
11*				41.1	1.307	31.46	30.15		

* Solution of 30 mm KOH.

TABLE IX a.
Summary of Results on Horse Hemoglobin No. H 58. June 14, 1923.
Total [Hb] = 7.08 mm per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$
	[HbO ₂]	[BHb]	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	[HbO ₂]	[BHb]	$\frac{[\text{BHb}_2]}{[\text{Hb}_2]}$	
	mm	m.-Eq.	m.-Eq.	mm	m.-Eq.	m.-Eq.	m.-Eq.
6.8	5.60 (7.08)*	2.15 (2.73)	(0.38)	0.05 (0.00)	-0.03 (-0.05)	(-0.007)	0.393
7.0	5.73 (7.08)	6.80 (7.55)	(1.07)	0.05 (0.00)	3.65 (3.62)	(0.51)	0.555
7.2	5.83 (7.08)	11.10 (11.91)	(1.68)	0.10 (0.00)	7.40 (7.34)	(1.04)	0.646
7.4	5.90 (7.08)	15.0 (15.81)	(2.23)	0.20 (0.00)	11.10 (10.96)	(1.55)	0.684
7.6	5.95 (7.08)	19.1 (19.98)	(2.82)	0.40 (0.00)	14.8 (14.49)	(2.05)	0.775

pH	6.9	7.1	7.3	7.5
β_o	3.45	3.05	2.75	2.95
β_R	2.60	2.65	2.55	2.50

* The numbers enclosed in parentheses indicate values for complete oxygenation or reduction extrapolated from the experimental values directly above them.

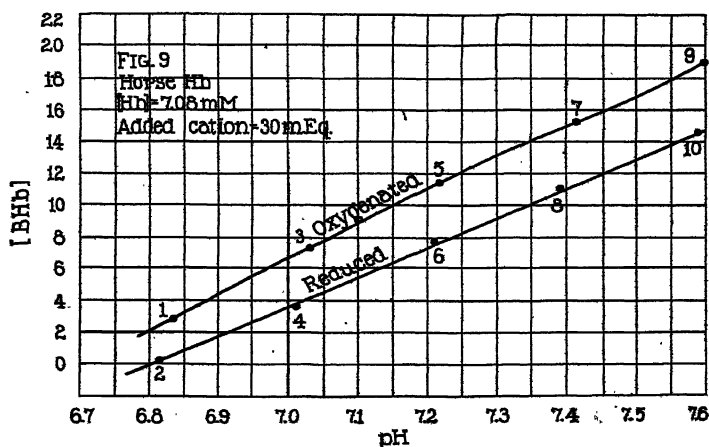


FIG. 9.

TABLE X a.

Summary of Results on Horse Hemoglobin No. H 51. May 4, 1923.

Total [Hb] = 6.50 mm per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$
	[HbO ₂]	[BHb]	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	[HbO ₂]	[BHb]	$\frac{[\text{BHb}_R]}{[\text{Hb}_R]}$	
	mM	m.-Eq.	m.-Eq.	mM	m.-Eq.	m.-Eq.	m.-Eq.
6.8	5.12 (6.87)*	2.3 (2.71)	(0.39)	0.4 (0.00)	1.2 (0.3)	(0.04)	0.233
7.0	5.33 (6.87)	7.2 (7.91)	(1.15)	0.8 (0.00)	5.1 (4.73)	(0.69)	0.464
7.2	5.45 (6.87)	11.50 (12.43)	(1.81)	0.87 (0.00)	8.5 (7.93)	(1.15)	0.653
7.4	5.50 (6.87)	15.0 (15.94)	(2.32)	0.40 (0.00)	11.5 (11.23)	(1.63)	0.686
7.6	5.54 (6.87)	18.1 (18.94)	(2.75)	0.33 (0.00)	14.8 (14.59)	(2.12)	0.634
7.8	5.54 (6.87)	20.0 (21.61)	(3.15)	0.60 (0.00)	18.3 (17.98)	(3.62)	0.596
8.0	5.45 (6.87)	23.4 (23.95)	(3.54)	1.10 (0.00)	21.7 (21.27)	(3.10)	0.301

pH	6.9	7.1	7.3	7.5	7.7	7.9
β_o	3.8	3.3	2.65	2.15	2.0	1.95
β_R	3.25	2.3	2.4	2.45	2.5	2.4

* The numbers enclosed in parentheses indicate values for complete oxygenation or reduction extrapolated from the experimental values directly above them.

TABLE X.

Experiment on Horse Hemoglobin No. H 51. May 4, 1923.

Total H₂O = 909.1 gm. per liter.

" Hb = 6.50 mm " "

" HbO₂ = 5.6 " " "

" KOH = 50.0 " " "

 $\alpha_{\text{CO}_2} = 0.555 \times 0.9091 = 0.504$ H₂CO₃ = $p_{\text{CO}_2} \times 0.02964$

pK' = 6.17

No.	P _{O2}	Total [O ₂].	[HbO ₂]	P _{CO2}	[H ₂ CO ₃]	Total [CO ₂].	[BHCO ₃]	pH	[BHb]
	mm.	mm	mm	mm.	mm	mm	mm		m.-Eq.
1	(135)	5.28	5.12	355.8	10.54	58.20	47.66	6.825	2.84
						57.85	47.31	6.822	2.69
2	(0)	0.38	0.38	391.1	11.60	60.57	48.97	6.795	1.03
				392.3	11.63		48.94	6.793	1.06
3	(135)	5.59	5.43	157.1	4.658	44.97	40.31	7.107	9.69
						45.08	40.42	7.108	9.58
4	(0)	1.07	1.07	178.9	5.301	48.85	43.55	7.078	6.45
5	(135)	4.63	4.47	66.7	1.977	36.93	34.95	7.417	15.05
6	(0)	0.37	0.37	77.6	2.30	40.85	38.55	7.394	11.45
7	(135)	5.72	5.56	27.4	0.812	30.65	29.84	7.735	20.16
8	(0)	0.30	0.30	34.2	1.014	34.60	33.59	7.690	16.44
9	(135)	5.58	5.42	11.6	0.344	26.28	25.94	8.049	24.06
10	(0)	1.17	1.17	12.9	0.382	28.35	27.97	8.034	22.03
						28.18	27.80	8.032	22.20

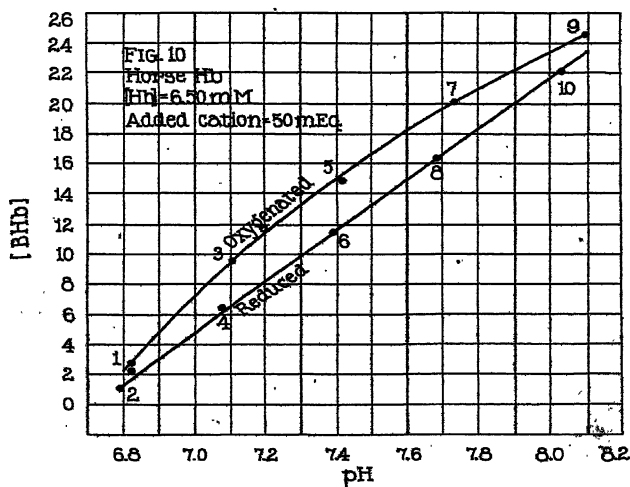


FIG. 10.

TABLE XI a.

Summary of Results on Horse Hemoglobin No. H 70. Feb. 5,

Total [Hb] = 8.00 mM per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$
	[HbO ₂]	[BHb]	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	[HbO ₂]	[BHb]	$\frac{[\text{BHb}_R]}{[\text{Hb}_R]}$	
	mM	m.-Eq.	m.-Eq.	mM	m.-Eq.	m.-Eq.	m.-Eq.
6.80	6.23 (8.00)*	3.80 (4.60)	(0.57)	0.08 (0.00)	1.0 (0.96)	(0.12)	0.45
7.00	6.28 (8.00)	8.20 (9.26)	(1.16)	0.09 (0.00)	4.5 (4.45)	(0.56)	0.598
7.20	6.60 (8.00)	12.90 (13.79)	(1.72)	0.15 (0.00)	8.8 (8.7)	(1.09)	0.636
7.40	6.68 (8.00)	17.7 (18.66)	(2.33)	0.22 (0.00)	13.5 (13.36)	(1.67)	0.650
7.60	6.66 (8.00)	21.9 (22.71)	(2.84)	0.38 (0.00)	18.1 (17.87)	(2.23)	0.605
7.80	6.49 (8.00)	25.6 (26.46)	(3.31)	0.72 (0.00)	22.3 (21.89)	(2.74)	0.572
8.00	6.51 (8.00)	28.7 (29.44)	(3.68)	1.13 (0.00)	26.0 (25.43)	(3.18)	0.50

pH	6.9	7.1	7.3	7.5	7.7	7.9
β_o	2.95	2.80	3.05	2.65	2.35	1.85
β_R	2.20	2.65	2.90	2.80	2.55	2.20

* The numbers enclosed in parentheses indicate values for complete oxygenation or reduction extrapolated from the experimental values directly above them.

TABLE XI.*

Experiment on Horse Hemoglobin No. H 70. Feb. 5, 1924.

Total H_2O = 892.3 gm. per liter." Hb = 8.00 mm " "" HbO_2 = 6.73 " " "" KOH = 50.0 " " " $\alpha_{CO_2} = 0.555 \times 0.8923 = 0.4951$ $H_2CO_3 = p_{CO_2} \times 0.02908$ $pK' = 6.17$

No.	P_{O_2}	Total $[O_2]$	$[HbO_2]$	P_{CO_2}	$[H_2CO_3]$	Total $[CO_2]$	$[BHCO_3]$	pH	$[BHb]$
	mm.	mm	mm	mm.	mm	mm	mm		m.-Eq.
1	(4.30)	6.86	6.23	276.6	8.048	52.05	44.00	6.908	6.00
2	(0)	0.08	0.08	296.2	8.62	56.02	47.40	6.910	2.60
						55.80	47.18	6.908	2.82
3	(160)	6.56	6.32	159.4	4.64	44.07	39.43	7.100	10.57
4	(0)	0.10	0.10	173.0	5.034	48.52	43.49	7.107	6.51
5	(135)	6.30	6.14	107.6	3.128	39.44	36.31	7.235	13.69
6	(0)	0.21	0.21	96.9	2.82	41.20	38.38	7.305	11.62
7	(135)	6.68	6.52	52.7	1.533	32.30	30.77	7.473	19.23
8	(0)	0.23	0.23	57.8	1.681	36.36	34.68	7.485	15.32
9	(135)	6.64	6.48	24.5	0.713	26.35	25.64	7.726	24.36
10	(0)	0.52	0.52	29.7	0.864	30.33	29.47	7.703	20.53
11	(137.5)	6.67	6.51	10.0	0.291	21.10	20.81	8.025	29.19
12	(2.8)	1.33	1.33	7.1	0.207	21.31	21.10	8.178	28.90

* Our thanks are due to Dr. Cecil Murray, whose assistance enabled us to add this experiment to the series.

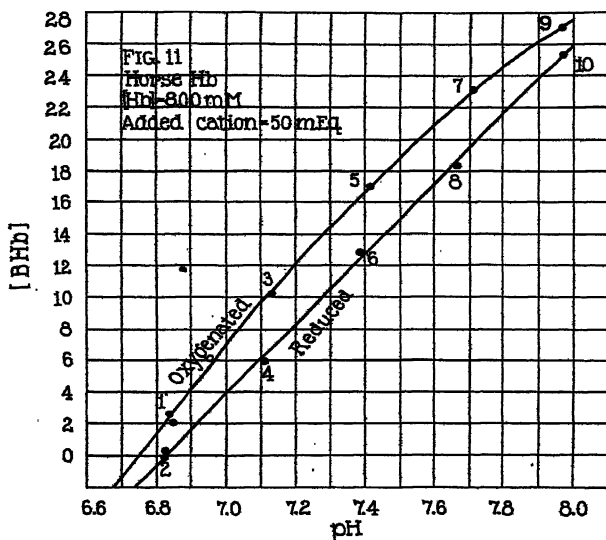


FIG. 11.

TABLE XII.

*Experiment on Horse Hemoglobin No. H 55. May 16, 1923.*Total H_2O = 896.5 gm. per liter.

" Hb = 7.39 mm " "

" HbO_2 = 6.09 " " "

" KOH = 30.00 " " "

" KCl = 115.00 " " "

 $\alpha_{CO_2} = 0.555 \times 0.8965 = 0.4974$ $H_2CO_3 = p_{CO_2} \times 0.02922$ $pK' = 6.11$

No.	P_{O_2}	Total [O_2].	[HbO_2]	P_{CO_2}	[H_2CO_3]	Total [CO_2].	[$BHCO_3$]	pH	[BHb]
	mm.	mm	mm	mm.	mm	mm	mm		m.-H ₂ .
1	(135)	5.39	5.15	200.8	5.867	33.55	27.68	6.783	2.32
2	(0)	0.07	0.07	233.1	6.809	37.60	30.79	6.765	-0.79
3	(135)	5.55	5.39	107.7	3.147	26.28	23.13	6.977	6.87
4	(0)	0.02	0.02	131.9	3.856	30.82	26.96	6.954	3.04
5	(135)	5.78	5.62	55.1	1.610	19.91	18.30	7.166	11.70
6	(0)	0.03	0.03	71.9	2.103	24.93	22.83	7.146	7.17
7	(135)	5.79	5.63	27.3	0.7980	14.82	14.02	7.355	15.98
8	(0)	0.06	0.06	38.3	1.120	19.90	18.78	7.334	11.22
9	(135)	5.79	5.63	12.7	0.3712	10.67	10.30	7.553	19.70
10	(0)	0.03	0.03	20.0	0.5747	15.40	14.83	7.522	15.17
11*				41.1	1.307	30.90	29.59		

* Solution of 30 mm KOH + 115 mm KCl.

TABLE XII a.

Summary of Results on Horse Hemoglobin No. H 55. May 16, 1923.

Total [Hb] = 7.39 mm per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$
	[HbO ₂]	[BHb]	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	[HbO ₂]	[BHb]	$\frac{[\text{BHb}_R]}{[\text{Hb}_R]}$	
	mm	m.-Eq.	m.-Eq.	mm	m.-Eq.	m.-Eq.	m.-Eq.
6.8	5.17 (7.39)*	2.6 (3.76)	(0.51)	0.07 (0.00)	-0.08 (-0.12)	(-0.02)	0.526
7.0	5.45 (7.39)	7.60 (8.89)	(1.20)	0.02 (0.00)	4.00 (3.99)	(0.54)	0.663
7.2	5.58 (7.39)	12.45 (13.81)	(1.87)	0.05 (0.00)	8.30 (8.26)	(1.118)	0.750
7.4	5.63 (7.39)	16.7 (17.99)	(2.43)	0.05 (0.00)	12.60 (12.56)	(1.70)	0.735
7.6	5.63 (7.39)	20.55 (21.73)	(2.94)	0.03 (0.00)	16.80 (16.78)	(2.27)	0.670

pH	6.9	7.1	7.3	7.5
β_o	3.45	3.35	2.80	2.55
β_R	2.8	2.9	2.9	2.85

* The numbers enclosed in parentheses indicate values for complete oxygenation or reduction extrapolated from the experimental values directly above them.

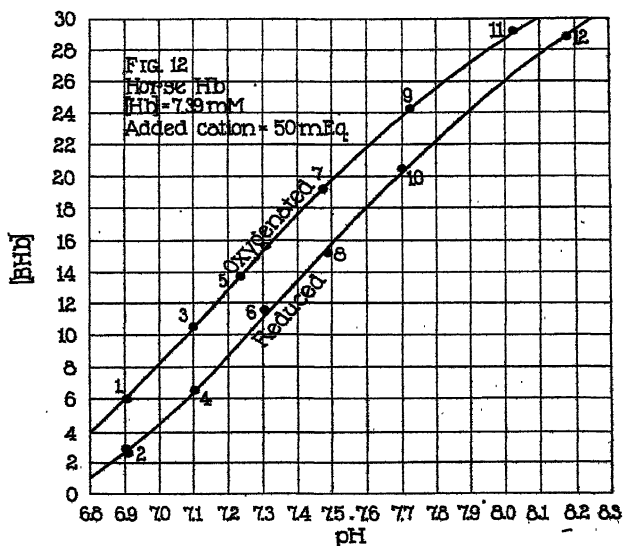


FIG. 12.

TABLE XIII.

*Experiment on Horse Hemoglobin No. H 56. June 1, 1923.*Total H_2O = 924.0 gm. per liter.

" Hb = 5.11 mM " "

" HbO_2 = 4.55 " " "

" KOH = 30.0 " " "

" KCl = 115.0 " " "

 $\alpha_{CO_2} = 0.555 \times 0.924 = 0.513$ $H_2CO_3 = p_{CO_2} \times 0.03013$ $pK' = 6.17$

No.	P_{O_2}	Total [O ₂]	[HbO ₂]	P_{CO_2}	[H ₂ CO ₃]	Total [CO ₂]	[BHCO ₃]	pH	[BHb]
	mm.	mM	mM	mm.	mM	mM	mM		m.-Eq.
1	(135)	4.11	3.95	201.4	6.068	34.69	28.62	6.784	1.38
2	(0)	0.09	0.09	233.5	7.036	37.81	30.77	6.751	-0.77*
3	(135)	4.30	4.14	98.5	2.967	27.42	24.45	7.026	5.55
4	(0)	0.10	0.10	131.4	3.960	31.83	27.87	6.958	2.13
5	(135)	4.33	4.17	56.6	1.705	23.09	21.38	7.280	8.62
6	(0)	0.14	0.14	71.6	2.157	26.80	24.64	7.168	5.36
7	(135)	4.44	4.28	28.7	0.8648	18.90	18.04	7.429	11.96
8	(0)	0.23	0.23	34.2	1.031	21.84	20.81	7.415	9.19
9	(135)	4.61	4.45	11.3	0.340	14.48	14.14	7.729	15.86
10	(0)	0.25	0.25	18.8	0.5665	18.53	17.96	7.611	12.04
11†				41.2	1.31	30.83	29.52		

* $HbHCO_3$.

† Solution of 30 mM KOH + 115 mM KCl.

TABLE XIII a.

Summary of Results on Horse Hemoglobin No. H 56. June 1, 1923.

Total [Hb] = 5.11 mm per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$
	[HbO ₂]	[BHb]	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	[HbO ₂]	[BHb]	$\frac{[\text{BHb}_R]}{[\text{Hb}_R]}$	
	mm	m.-Eq.	m.-Eq.	mm	m.-Eq.	m.-Eq.	m.-Eq.
6.8	4.00 (5.11)*	1.68 (2.18)	(0.42)	0.09 (0.00)	-0.08 (-0.12)	(-0.02)	0.450
7.0	4.08 (5.11)	5.15 (5.74)	(1.12)	0.11 (0.00)	2.88 (2.82)	(0.55)	0.572
7.2	4.18 (5.11)	8.55 (9.17)	(1.80)	0.15 (0.00)	5.88 (5.78)	(1.13)	0.663
7.4	4.28 (5.11)	11.58 (12.06)	(2.36)	0.21 (0.00)	8.88 (8.74)	(1.71)	0.664
7.6	4.37 (4.37)	14.28 (14.65)	(2.87)	0.25 (0.00)	11.90 (11.76)	(2.30)	0.578

pH	6.9	7.1	7.3	7.5
β_o	3.5	3.4	2.8	2.6
β_R	2.9	2.9	2.9	

*The numbers enclosed in parentheses indicate values for complete oxygenation or reduction extrapolated from the experimental values directly above them.

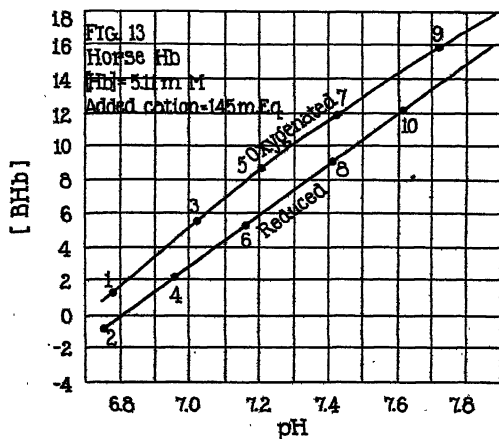


FIG. 13.

TABLE XIV.

Experiment on Dog Hemoglobin No. D 22. May 15, 1923.

Total H_2O = 932.0 gm. per liter.

" Hb = 5.19 mm " "

" HbO_2 = 4.32 " " "

" NaOH = 30.0 " " "

 $\alpha_{CO_2} = 0.555 \times 0.932 = 0.5173$ $H_2CO_3 = p_{CO_2} \times 0.0304$ $pK' = 6.17$

No.	P_{O_2}	Total $[O_2]$	$[HbO_2]$	P_{CO_2}	$[H_2CO_3]$	Total $[CO_2]$	$[BHCO_3]$	pH	$[BHb]$
	mm.	mm	mm	mm.	mm	mm	mm		m.-Eq.
1	(135)	4.11	3.95	212.1	6.448	34.45	28.00	6.808	2.00
2	(0)	0.14	0.14	232.2	7.060	36.90	29.84	6.796	-0.16
3	(135)	4.19	4.03	118.1	3.590	29.22	25.63	7.024	4.37
4	(0)	0.12	0.12	132.9	4.040	31.49	27.45	7.002	2.55
5	(135)	4.25	4.09	63.6	1.933	24.70	22.77	7.241	7.23
6	(0)	0.13	0.13	75.7	2.301	27.65	25.35	7.212	4.65
7	(135)	4.33	4.17	33.8	1.027	21.40	20.37	7.468	9.63
8	(0)	0.12	0.12	41.6	1.265	24.37	23.10	7.432	6.90
9*				41.2	1.310	31.05	29.74		

* Solution of 30 mm NaOH.

TABLE XIV a.

Summary of Results on Dog Hemoglobin No. D 22. May 15, 1923.

Total $[Hb] = 5.19$ mm per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{[BHb]}{[O_2Hb]}$
	$[HbO_2]$	$[BHb]$	$\frac{[BHbO_2]}{[HbO_2]}$	$[HbO_2]$	$[BHb]$	$\frac{[BHb]}{[Hb]}$	
	mm	m.-Eq.	m.-Eq.	mm	m.-Eq.	m.-Eq.	m.-Eq.
6.8	3.95 (5.19)*	1.90 (2.45)	(0.47)	0.14 (0.00)	0.22 (0.16)	(0.03)	0.441
7.0	4.03 (5.19)	4.27 (4.82)	(0.93)	0.12 (0.00)	2.43 (2.37)	(0.46)	0.471
7.2	4.09 (5.19)	6.65 (7.25)	(1.40)	0.13 (0.00)	4.50 (4.43)	(0.85)	0.543
7.4	4.17 (5.19)	9.0 (9.60)	(1.85)	0.12 (0.00)	6.60 (6.53)	(1.26)	0.593

pH	6.9	7.1	7.3
β_o	2.3	2.35	2.25
β_r	2.15	1.95	2.05

* The numbers enclosed in parentheses indicate values for complete oxygenation or reduction extrapolated from the experimental values directly above them.

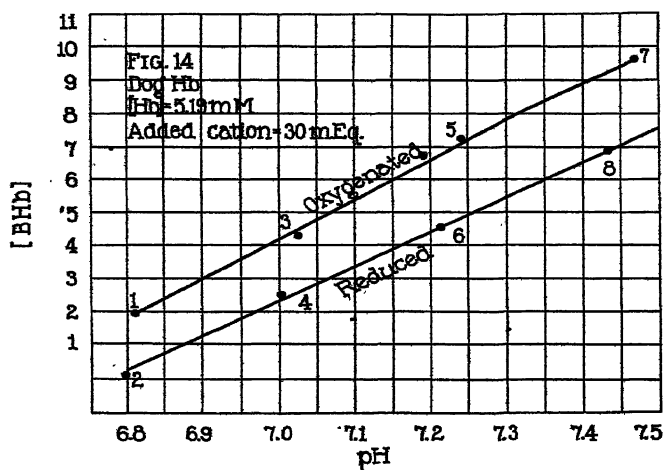


FIG. 14.

TABLE XV.

*Experiment on Dog Hemoglobin No. D 23. May 24, 1923.*Total H_2O = 942.3 gm. per liter.

" Hb = 4.3 mm " "

" HbO_2 = 3.82 " " "

" NaOH = 30.0 " " "

 $\alpha_{\text{CO}_2} = 0.555 \times 0.942 = 0.5228$ $\text{H}_2\text{CO}_3 = p_{\text{CO}_2} \times 0.03073$ $\text{pK}' = 6.17$

No.	P_{O_2}	Total $[\text{O}_2]$	$[\text{HbO}_2]$	P_{CO_2}	$[\text{H}_2\text{CO}_3]$	Total $[\text{CO}_2]$	$[\text{BHCO}_2]$	pH	$[\text{BHb}]$
	mm.	mm	mm	mm.	mm	mm	mm		m.-Eq.
1	(135)	3.65	3.49	212.6	6.528	34.70	28.17	6.805	1.83
2	(0)	0.16	0.16	234.8	7.214	37.69	30.48	6.796	-0.48
3	(135)	3.84	3.68	117.8	3.620	30.58	26.96	7.042	3.04
4	(0)	0.08	0.08	131.6	4.042	33.19	29.15	7.028	0.85
5	(135)	3.85	3.69	64.1	1.970	26.58	24.61	7.267	5.39
6	(0)	0.13	0.13	75.2	2.310	29.06	26.75	7.234	3.25
7	(135)	3.86	3.70	*34.2	1.051	23.50	62.45	7.499	7.55
8	(0)	0.13	0.13	44.6	1.371	26.70	25.33	7.436	4.67
9				40.5	1.264	31.34	30.08		

TABLE XV a.

*Summary of Results on Dog Hemoglobin No. D 23. May 24, 1923.*Total $[\text{Hb}] = 4.3$ mm per liter.

pH	Oxygenated solutions.			Reduced solutions.			$\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$
	$[\text{HbO}_2]$	$[\text{BHb}]$	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	$[\text{HbO}_2]$	$[\text{BHb}]$	$\frac{[\text{BHb}_2]}{[\text{Hb}_2]}$	
	mm	m.-Eq.	m.-Eq.	mm	m.-Eq.	m.-Eq.	m.-Eq.
6.8	3.49 (4.30)*	0.7 (0.97)	(0.23)	0.16 (0.00)	-0.4 (-0.45)	(-0.1)	0.33
7.0	3.68 (4.30)	2.65 (2.91)	(0.68)	0.14 (0.00)	1.15 (1.09)	(0.25)	0.424
7.2	3.69 (4.30)	4.60 (4.91)	(1.14)	0.13 (0.00)	2.77 (2.70)	(0.63)	0.514
7.4	3.70 (4.30)	6.60 (7.07)	(1.64)	0.13 (0.00)	4.4 (4.32)	(1.01)	0.616

pH	6.9	7.1	7.3
β_{O}	2.25	2.3	2.5
β_{R}	1.75	1.9	1.9

*The numbers enclosed in parentheses indicate values for complete oxygenation or reduction extrapolated from the experimental values directly above them.

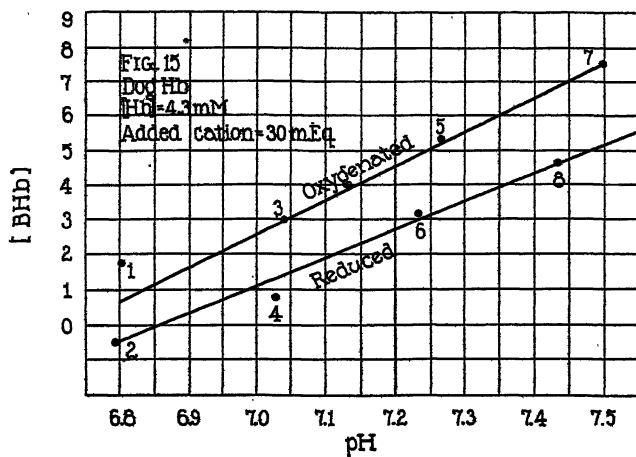


FIG. 15.

In Tables I *a*, II *a*, etc., we have calculated from interpolations on the [BHb], pH curves at 0.2 pH intervals the equivalents of base bound per molecule of reduced and oxygenated hemoglobin, and thence the molecular buffer values β_0 and β_R , and the increase of base bound per mol of hemoglobin in passing from the reduced to the oxygenated state at constant pH, symbolized as $\Delta[\text{BHb}]$.
as $\Delta[\text{O}_2\text{Hb}]$

The values obtained for $\frac{[\text{BHb}]}{[\text{Hb}]}$ (the base bound per mol of total hemoglobin), for the buffer values β_0 and β_R , and for the increase $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ in equivalents of base bound per mol of oxygen combined, are summarized from all the experiments in Tables XVI, XVII, and XVIII, respectively. The "calculated" values given in Tables XVI and XVIII for comparison with the average observed values of [BHb] and $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ are estimated from Equations 20 and 8, which are developed below.

The average values obtained for the base bound by reduced horse hemoglobin, as indicated in Table XVI, are plotted in Fig. 16.

The data presented lead directly to the following conclusions.

(1) The isoelectric point of reduced horse hemoglobin, defined as the pH at which hemoglobin binds equivalent amounts of acid and base, lies at $\text{pH } 6.81 \pm 0.02$.

(2) The molecular buffer value β_R of reduced hemoglobin, measured as $\frac{d[\text{BHb}_R]}{[\text{Hb}]_R dp\text{H}}$ (Van Slyke (8)), is nearly constant between the isoelectric point and pH 7.6. As seen from Table XVII the β_R values tend to be a little higher near the isoelectric point, and a little lower above pH 7.3. Fig. 16 indicates, however, that the [BHb]_R, pH curve may be represented between pH 6.8 and 7.6 for a given solution as a straight line of constant slope (buffer value) which deviates but slightly from the observed

(3) β_R , though nearly constant for a given solution, is definitely increased by increasing the cation concentration (Table XVII and Fig. 16).

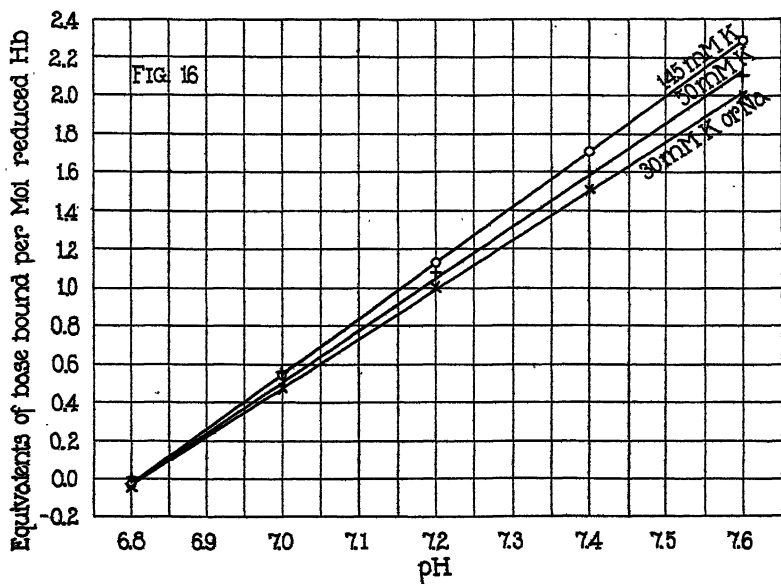


FIG. 16. Average values for base bound by reduced horse hemoglobin in solutions with different concentrations of monovalent cation.

TAB. XV.
Summary. *Equivalents of Base Bound per Mol of Hemoglobin.*

Hemoglobin and electrolyte.	Experiment No.	pH = 6.8		pH = 7.0		pH = 7.2		pH = 7.4		pH = 7.6	
		$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	$\frac{[\text{BHb}_2]}{[\text{Hb}_2]}$	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	$\frac{[\text{BHb}_2]}{[\text{Hb}_2]}$	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	$\frac{[\text{BHb}_2]}{[\text{Hb}_2]}$	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	$\frac{[\text{BHb}_2]}{[\text{Hb}_2]}$	$\frac{[\text{BHbO}_2]}{[\text{HbO}_2]}$	$\frac{[\text{BHb}_2]}{[\text{Hb}_2]}$
Horse Hb. 30 mm [NaHb] + [NaHCO ₂]	1										
	2	0.38	-0.13	0.99	0.43	1.54	0.94	2.15	1.46	2.78	2.01
	3	0.38	-0.01	1.02	0.53	1.61	1.00	2.24	1.57	2.61	1.93
	4	0.23	-0.21	0.88	0.36	1.53	0.91	2.10	1.47	2.55	1.82
	5	0.18	-0.34	0.88	0.28	1.49	0.80	2.02	1.32	2.70	1.98
	6	0.41	-0.07	1.01	0.48	1.61	1.00	2.20	1.54		
Horse Hb. 30 mm [KHb] + [KHCO ₂]	7	0.43	-0.05	1.04	1.46	1.59	0.98	2.09	1.46		
	8	0.47	-0.00	1.03	0.49	1.58	1.01	2.13	1.51		
	9	0.38	-0.01	1.07	0.51	1.68	1.04	2.23	1.55	2.82	2.05
	Average*.. Calculated†	0.39	-0.04	1.03	0.48	1.61	1.01	2.18	1.52	2.77	2.01
		0.41	-0.03	1.02	0.49	1.62	1.01	2.20	1.53	2.74	2.05
Horse Hb. 50 mm [KHb] + [KHCO ₂]	10	0.39	0.04	1.15	0.69	1.81	1.15	2.32	1.63	2.75	2.12
	11	0.57	0.12	1.16	0.56	1.72	1.09	2.33	1.67	2.84	2.23
	Average*.. Calculated†	0.48	0.08	1.16	0.62	1.79	1.12	2.33	1.65	2.80	2.17
		0.45	-0.03	1.07	0.51	1.67	1.05	2.23	1.59	2.75	2.13

Horse Hb. 30 mm [K ₂ Hb] + [KHCO ₃] + 115 mm [KCl]	12	0.51	(-0.02)	1.20	0.54	1.87	1.12	2.43	1.70	2.94	2.27
	13	0.42	(-0.02)	1.12	0.55	1.80	1.13	2.36	1.71	2.87	2.30
	Average... Calculated†	0.47 0.54	(-0.02) -0.03	1.16 1.20	0.55 0.54	1.83 1.82	1.13 1.13	2.40 2.40	1.71 1.71	2.90 2.93	2.29 2.29
Dog Hb. 30 mm [NaHb] + [NaHCO ₃]	14	0.47	0.03	0.93	0.46	1.40	0.85	1.85	1.26		
	15	0.23	-0.10	0.88	0.25	1.14	0.63	1.64	1.01		
	Average... Calculated†	0.35	-0.03	0.80	0.35	1.27	0.74	1.75	1.14		

* Nos. 4 and 5 are excluded from average.

† Calculated values are by Equation 20, with numerical constants from Table XXIII.

(4) From Table XVII it is seen that β_0 the buffer value of *oxyhemoglobin*, is highest at pH 6.9, and decreases steadily with increasing pH.

(5) The increase $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ in base-binding power caused by

TABLE XVII.

Summary. Buffer Values of Reduced and Oxygenated Hemoglobin.

Hemoglobin and electrolyte.	Experiment No.	pH = 6.9		pH = 7.1		pH = 7.3		pH = 7.5		pH = 7.7		pH = 7.9	
		β_0	β_R	β_0	β_R	β_0	β_R	β_0	β_R	β_0	β_R	β_0	β_R
Horse Hb. 30 mm [NaHb] + [NaHCO ₃]	1					3.1	2.6						
	2	3.1	2.8	3.1	2.9	3.2	2.9						
	3	3.2	2.7	3.1	2.6	3.0	2.6	2.8	2.3				
	4	3.3	2.9	3.3	2.8	2.9	2.8	2.9	2.4				
	5	3.5	3.1	3.1	2.6	2.7	2.6	2.7	2.5				
	6	3.7	2.9	2.9	2.5	2.8	2.7						
Horse Hb. 30 mm [KHb] + [KHCO ₃]	7	3.1	2.6	2.8	2.6	2.5	2.4						
	8	3.7	2.6	2.8	2.5	2.8	2.7						
	9	3.5	2.6	3.1	2.7	2.8	2.6	3.0	2.5				
	Average.	3.3	2.8	3.0	2.6	2.7	2.6	2.8	2.4				
Horse Hb. 50 mm [KHb] + [KHCO ₃]	10	3.8	3.3	3.3	2.3	2.7	2.4	2.2	2.5	2.0	2.5	2.0	2.4
	11	3.0	2.2	2.8	2.7	3.1	2.9	2.7	2.8	2.4	2.6	1.9	2.2
	Average.	3.4	2.8	3.0	2.5	2.9	2.7	2.5	2.7	2.2	2.6	1.95	2.3
Horse Hb. 30 mm [KHb] + [KHCO ₃] + 115 mm [KCl]	12	3.5	2.8	3.4	2.9	2.8	2.9	2.6	2.9				
	13	3.5	2.9	3.4	2.9	2.8	2.9	2.6	2.9				
	Average.	3.5	2.9	3.4	2.9	2.8	2.9	2.6	2.9				
Dog Hb. 30 mm [NaHb] + [NaHCO ₃]	14	2.3	2.2	2.4	2.0	2.3	2.1						
	15	2.3	1.8	2.3	1.9	2.5	1.9						
	Average.	2.3	2.0	2.4	2.0	2.4	2.0						

oxygenation of hemoglobin has a maximum value of about 0.7 equivalent of base per mol of hemoglobin, and this maximum occurs at a definite pH. The value of $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ diminishes as the pH is removed in either direction from this point.

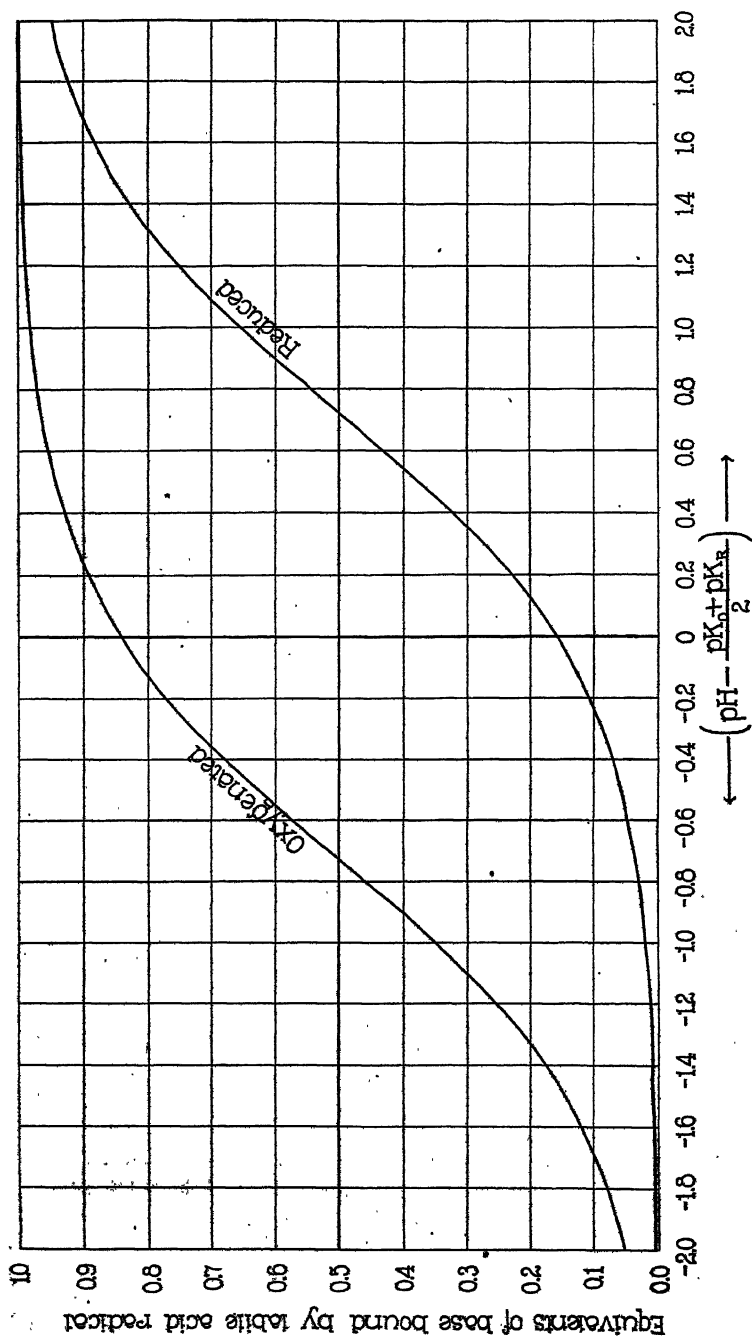


Fig. 17. Curves for graphic calculation of pK'_a and pK_b from observed $\frac{d[\text{B}]}{d[\text{H}^+]}$ and pH values.

TABLE XVIII.
Summary of $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ Values.

Hemoglobin and electrolyte.	Experiment No.	Values of $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ at pH:						
		6.8	7.0	7.2	7.4	7.6	7.8	8.0
Horse Hb. 30 mM [NaHb] + [NaHCO ₃]	1			0.60	0.69			
	2	0.51	0.56	0.61	0.67			
	3	0.39	0.49	0.58	0.66	0.77		
	4	0.44	0.52	0.61	0.63	0.66		
	5	0.33	0.56	0.62	0.65			
	6	0.43	0.60	0.69	0.69	0.73		
Horse Hb. 30 mM [KHb] + [KHCO ₃]	7	0.50	0.58	0.61	0.63			
	8	0.48	0.53	0.57	0.63			
	9	0.39	0.56	0.65	0.68	0.78		
	Average....	0.43	0.55	0.61	0.66	0.74		
	Calculated.*	0.43	0.53	0.61	0.66	0.68		
Horse Hb. 50 mM [KHb] + [KHCO ₃]	10	0.23	0.46	0.65	0.69	0.63	0.53	0.39
	11	0.45	0.60	0.64	0.65	0.61	0.57	0.50
	Average....	0.34	0.53	0.65	0.67	0.62	0.55	0.45
	Calculated.*	0.48	0.56	0.62	0.64	0.62	0.56	0.48
Horse Hb. 30 mM [KHb] + [KHCO ₃] + 115 mM [KCl]	12	0.53	0.66	0.75	0.74	0.67		
	13	0.45	0.57	0.66	0.66	0.58		
	Average....	0.49	0.61	0.70	0.70	0.62		
	Calculated.*	0.57	0.64	0.68	0.68	0.64		
Dog Hb. 30 mM [NaHb] + [NaHCO ₃]	14	0.44	0.47	0.54	0.59			
	15	0.33	0.42	0.51	0.62			
	Average....	0.38	0.45	0.53	0.60			
	Calculated.*	0.37	0.44	0.54	0.60			

* The calculations are made by Equation 13, with the pK_a and pK_b values of Table XX.

(6) The pH of maximum $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ is lower in solutions of greater salt concentration, being approximately 7.6 in solutions of 30 mM cation concentration, 7.3 in those of 145 mM cation concentration.

The significance of these relationships will be discussed below.

Calculation of the Labile Acid Dissociation Constants of Reduced and Oxygenated Hemoglobin.

L. J. Henderson (6) has suggested that the change in the CO_2 capacity of blood with change in the degree of oxygenation is due to the fact that 1 acid hydrogen in the hemoglobin molecule (the amount of hemoglobin combining with 1 molecule of O_2) has its dissociation constant increased by oxygenation and lowered by reduction of the hemoglobin. In order to ascertain whether such an assumption is compatible with our results we have compared the values of $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ observed over the pH range of our experiments with the values estimated on the basis of this assumption.

In general the base bound as the salt Ba by an acid group of concentration C and dissociation constant K is indicated by Henderson's mass law equation as

$$(2) \quad \frac{[\text{Ba}]}{C} = \frac{K'}{K' + [\text{H}^+]}$$

where $K' = \frac{K}{\gamma}$, and γ is the degree of dissociation of the salt Ba into B^+ and a' ions. If we assign to the separate acid groups in oxyhemoglobin the dissociation constants K_{O_1} , K_{O_2} , K_{O_3} , etc., the total base bound by oxyhemoglobin will be indicated as

$$(3) \quad \frac{[\text{BHbO}_2]}{[\text{Hb}]} = \frac{K'_{O_1}}{K'_{O_1} + [\text{H}^+]} + \frac{K'_{O_2}}{K'_{O_2} + [\text{H}^+]} + \frac{K'_{O_3}}{K'_{O_3} + [\text{H}^+]} \dots \dots$$

Similarly, for reduced hemoglobin

$$(4) \quad \frac{[\text{BHb}_R]}{[\text{Hb}]} = \frac{K'_{R_1}}{K'_{R_1} + [\text{H}^+]} + \frac{K'_{R_2}}{K'_{R_2} + [\text{H}^+]} + \frac{K'_{R_3}}{K'_{R_3} + [\text{H}^+]} \dots \dots$$

We obtain $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$, the increase in alkali-binding power that accompanies isohydronic change from reduced to oxygenated hemoglobin, at any given $[\text{H}^+]$, in Equation 5 by subtracting 4 from 3.

$$(5) \quad \frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]} = \frac{[\text{BHbO}_2]}{[\text{Hb}]} - \frac{[\text{BHb}_2]}{[\text{Hb}]} =$$

$$\left(\frac{K_{O_1}}{K_{O_1} + [\text{H}^+]} - \frac{K_{R_1}}{K_{R_1} + [\text{H}^+]} \right) + \left(\frac{K_{O_2}}{K_{O_2} + [\text{H}^+]} - \frac{K_{R_2}}{K_{R_2} + [\text{H}^+]} \right) + \dots$$

It is obvious that if K_{O_1} and K_{R_1} are equal, the difference between the terms in the first parenthesis, $\frac{K_{O_1}}{K_{O_1} + [\text{H}^+]} - \frac{K_{R_1}}{K_{R_1} + [\text{H}^+]}$ becomes zero. Similarly, the terms containing K_{O_2} and K_{R_2} cancel out, if K_{O_2} and K_{R_2} are equal; and so on. The only terms of Equation 5 that are not cancelled out are those in which K_{O_n} does not equal K_{R_n} ; i.e., those terms representing acid groups whose dissociation constants are changed by oxygenation and reduction.

Let us assume, as did Henderson, that oxygenation changes the dissociation constant of only one acid group, to which we assign the constants K'_O and K'_R for the oxygenated and reduced forms, respectively. Then by the cancellations above discussed Equation 5 simplifies to

$$(6) \quad \frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]} = \frac{K'_O}{K'_O + [\text{H}^+]} - \frac{K'_R}{K'_R + [\text{H}^+]}$$

$$(7) \quad = \frac{1}{1 + \frac{[\text{H}^+]}{K'_O}} - \frac{1}{1 + \frac{[\text{H}^+]}{K'_R}}$$

$$(8) \quad \frac{1}{1 + 10^{pK'_O - \text{pH}}} - \frac{1}{1 + 10^{pK'_R - \text{pH}}}$$

Equations 7 and 8 are obvious rearrangements of Equation 6.

The estimation of K'_O and K'_R from observed values of $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ and pH may be made algebraically by substituting two sets of

experimental values for $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ and $[\text{H}^+]$ in Equation 6 and solving for K_o and K_R , as was done by Henderson (6), or the problem may be solved graphically by using (1) two curves expressing the two terms in the right-hand member of Equation 8, together with (2) the fact that if the hypothesis expressed in Equation 8 is correct, the maximum value of $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ occurs at the point where $\text{pH} = \frac{\text{pK}'_o + \text{pK}'_R}{2}$, midway between pK'_o and pK'_R .

The fact that in general a maximum value of $[\text{Ha}]_1 - [\text{Ha}]_2$, hence of $[\text{Ba}]_1 - [\text{Ba}]_2$, occurs when $\text{pH} = \frac{\text{pK}'_1 + \text{pK}'_2}{2}$ appears obvious from inspection of a pair of curves like those of Fig. 17, and may be demonstrated as follows. We may write Henderson's equation as

$$(9) \quad [\text{Ha}]_1 = \frac{[\text{H}^+]C_1}{K'_1 + [\text{H}^+]}$$

$$(10) \quad [\text{Ha}]_2 = \frac{[\text{H}^+]C_2}{K'_2 + [\text{H}^+]}$$

where C is the total buffer concentration. $C_1 = C_2$, when equivalent concentrations of the two buffers are present. Letting $[\text{Ha}]_1 - [\text{Ha}]_2 = \Delta$, we have by subtraction

$$(11) \quad \Delta = \frac{[\text{H}^+]C}{K'_1 + [\text{H}^+]} - \frac{[\text{H}^+]C}{K'_2 + [\text{H}^+]}$$

Differentiating to obtain $\frac{d\Delta}{d[\text{H}^+]}$ and solving the value of $\frac{d\Delta}{d[\text{H}^+]}$ for zero to ascertain the maximum Δ , we find it occurs when

$$(12) \quad K_1 K_2 = [\text{H}^+]^2$$

In logarithmic terms this expression becomes

$$(13) \quad \text{pH} = \frac{\text{pK}'_1 + \text{pK}'_2}{2}$$

The graphic estimation of pK'_R and pK'_o is performed as follows. Two curves are constructed representing the general relationship of $\frac{[\text{Ba}]}{C}$ to $\text{pK}' - \text{pH}$ as calculated from the equation

$$(14) \quad \frac{[\text{Ba}]}{C} = \frac{1}{1 + 10^{pK' - \text{pH}}}$$

which is merely the logarithmic form of Equation 2. The curves are so constructed that they can be moved horizontally to both sides of any desired mid-point. With the pH of observed maximum $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ as the mid-point, the two curves are moved to the right and left of it by equal distances until the *vertical* distance between the curves measured at the mid-point equals the observed $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ value. The pH values of the intersections of the two curves with the horizontal line representing in each case $\frac{[\text{Ba}]}{C} = 0.5$ then indicate the pK'_O and pK'_R values. (For an acid buffer, $\text{pH} = \text{pK}'$ when $[\text{Ha}] = [\text{Ba}] = 0.5$. See Van Slyke (8), p. 542.) Similarly, the two curves may be so placed, still laterally equidistant from the same mid-point, that the vertical distances between them at other pH values correspond to the $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ values observed at those points. If both the experimental data and the assumption that only one acid buffer has its dissociation constant changed by oxygenation are correct, the curves remain in the same place and indicate the same pK_O and pK_R values, regardless of what points are used to locate them.

The values which we have used for the mid-point $\frac{\text{pK}'_R + \text{pK}'_O}{2}$ have been 7.60 for the hemoglobin solutions of 30 mM cation concentration, 7.40 for those of 50 mM, and 7.30 for those of 145 mM. For the solutions of 50 and 145 mM total cation concentration the points 7.40 and 7.30 correspond to maxima observed in $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$. For the solutions of 30 mM cation concentration a definite maximum was not determinable below pH 7.6, which marked the upper limit of pH reached in the experiments. We have assumed that the maximum did occur at pH 7.60, however, because the curve of the $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ values at varying pH within

the experimental range gave consistent values for pK'_o and pK'_R when this assumption was made. (Proof of the maximum and its location will be given by electrometric titrations in a later paper.)

Using the above values for $\frac{pK'_R + pK'_o}{2}$, viz. 7.6, 7.4, and 7.3, we have estimated, by the graphic method outlined, the values of pK'_R and pK'_o corresponding to the experimental $\frac{\Delta[BHb]}{\Delta[O_2Hb]}$ figures obtained at pH 6.8, 7.0, 7.2, 7.4, and 7.6 with each hemoglobin solution. With the solutions of 50 mM $[BHb] + [BHCO_3]$, content values up to pH 8.0 were obtained. The results are given in Table XIX.

In the individual $\frac{\Delta[BHb]}{\Delta[O_2Hb]}$, pH curves the data taken from the different pH points agree among themselves, in the $pK'_R - pK'_o$ figures yielded, as closely as could be expected from the experimental accuracy of the data. The closest agreement occurs among the data from pH 7.0, 7.2, and 7.4, where the experimental conditions were best. Because of the low CO_2 tensions at pH > 7.6, and the very high ones at pH 6.8, accuracy was more difficult than in the intermediate points. The lack of systematic variation in the $pK'_R - pK'_o$ values with varying pH is an indication that the variations are due to experimental error rather than to the manner of calculation.

The decline in pK' values with increase in cation concentration is to be expected. $pK'_a = -\log K_a + \log \gamma$, where K_a is the true dissociation constant of an acid and γ the activity or degree of dissociation of the salt. Since γ decreases with increasing cation concentration pK'_a must also decrease. Warburg (12) discusses the point, and shows that in general the empirical equation holds $pK'_a = pK_a - c\sqrt[3]{B}$ the value of c being 0.46 for sodium bicarbonate solutions. The decrease of pK'_R and pK'_o with increasing cation concentration is, therefore, consistent with the general behavior of solutions of salts of weak acids.

TABLE XIX
Values of pK'_R and pK'_O by Graphic Calculation from Experimental
 $\Delta[\text{BHb}]$
 $\Delta[\text{O}_2\text{Hb}]$ *Measurements.*

Experiment No.	Values of $pK'_R - pK'_O$ estimated from observed $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ at pH.							Average values			Remarks
	68	70	72	74	76	78	80	$pK'_R - pK'_O$	pK'_R	pK'_O	
1			1 56	1 41				1 48	8 34	6 86	Horse Hb. Cation concentration = 30 m.-Eq. $pK'_O + pK'_R = 7.6$ 2
2	1 72	1 56	1 44	1 45				1 56	8 38	6 82	
3	1 34	1 38	1 38	1 46	1 80			1 48	8 34	6 86	
4	1 48	1 46	1 48	1 38	1 40			1 44	8 32	6 88	
5	1 36	1 58	1 50	1 42				1 42	8 31	6 89	
6	1 50	1 52	1 46	1 48	1 60			1 51	8 36	6 84	
7	1 70	1 64	1 48	1 36				1 54	8 37	6 83	
8	1 64	1 48	1 36	1 36				1 46	8 33	6 87	
9	1 34	1 60	1 60	1 52	1 84			1 58	8 39	6 81	
Average of Experiments 1 to 9								1 50	8 35	6 85	
10	(0.64)*	1 06	1 45	1 38	1 40	1 32	1 10	1 29	8 05	6 75	Horse Hb. K = 50 m.-Eq. $pK'_O + pK'_R = 7.40$ 2
11	1.24	1 42	1 40	1.36	1 32	1 36	1 40	1 35	8 07	6 72	
Average of Experiments 10 and 11								1 32	8 06	6 74	
12	1.36	1 52	1 74	1 70	1 60			1 58	8 09	6 51	Horse Hb. K = 145 m.-Eq. $pK'_O + pK'_R = 7.3$ 2
13	1.16	1 28	1 40	1 40	1 30			1 31	7 96	6 64	
Average of Experiments 12 and 13								1 45	8 02	6 57	
14	1.60	1 28	1 28	1 24				1 26	8 23	6 97	Dog Hb. Na = 30 m.-Eq. $pK'_O + pK'_R = 7.6$ 2
15	1 16	1.61	1 26	1 32				1 22	8 21	6 99	
Average of Experiments 14 and 15								1 24	8 22	6 98	

* Value in parentheses not included in average.

On the other hand, that the relationship $pK'_R - pK'_O$ (or $\log \frac{K'_O}{K'_R}$) should be significantly altered with change in cation concentration could not be predicted by analogy with the known behavior of buffer acids in general, and does not appear to be demonstrated with certainty by our data. The average values of 1.50 and 1.45 for $\log \frac{K'_O}{K'_R}$ obtained from solutions of 30 and 145 m.-Eq. cation concentration, respectively, differ by a margin within the limits of experimental variation. The lower figure of 1.32 obtained from the two solutions with the intermediate

TABLE XX.

Values of pK'_R and pK'_O Estimated from Average $pK'_R - pK'_O$ Value of 1.46 for Horse Hemoglobin, 1.24 for Dog Hemoglobin.

Hemoglobin species.	Cation concentration.	$\frac{pK'_R + pK'_O}{2}$ (from pH of maximum $\frac{\Delta[BHb]}{\Delta[O_2Hb]}$ value).	pK'_R	pK'_O
	m.-Eq.			
Horse.	30	7.6	8.33	6.87
"	50	7.4	8.13	6.67
"	145	7.3	8.03	6.57
Dog.	30	7.6	8.22	6.98

50 m.-Eq. cation concentration may owe its difference from them to experimental conditions.

In estimating the pK'_R and pK'_O which we judge to approximate most closely the correct figures, we have accordingly used for horse hemoglobin a single constant $pK'_R - pK'_O$ value of 1.46 the average from all 13 experiments, rather than the three $pK'_R - pK'_O$ values obtained in Table XIX by averaging separately the data from solutions of 30, 50, and 145 m.-Eq. cation concentration. The pK'_R and pK'_O values thus calculated are given in Table XX.

From Table XVIII and Fig. 18 it appears that the observed $\frac{\Delta[BHb]}{\Delta[O_2Hb]}$ values agree within the limits of experimental accuracy

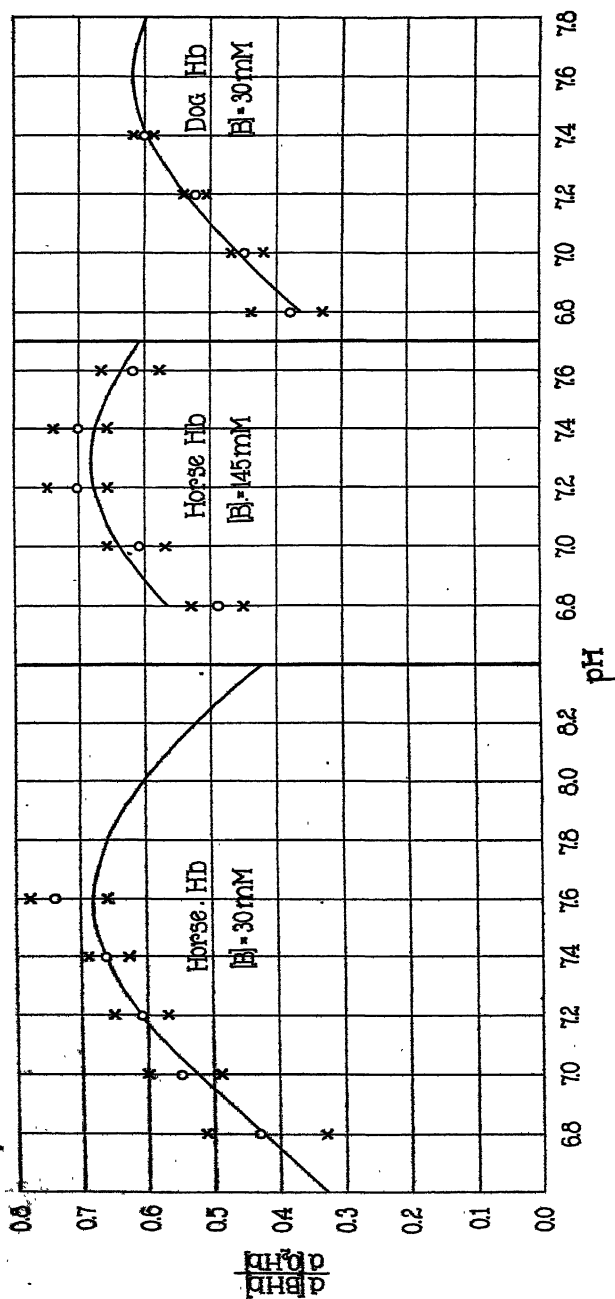


Fig. 18. Observed and calculated values of $\frac{d[BHb]}{d[O_2Hb]}$. The curves represent the values calculated by Equation 8. The X marks represent maximum and minimum experimental values; O marks, the average experimental values.





with those predictable according to the assumption that combination with a molecule of oxygen increases 29-fold ($\log \frac{K'_O}{K'_R} = 1.46 = \log 29$) the dissociation constant of one acid group in the hemoglobin molecule of the horse, 17-fold in that of the dog. From the CO_2 absorption curve of Christiansen, Douglas, and Haldane (2) on reduced and oxygenated human blood Henderson (6) estimated by means of simultaneous equations a value of 9 for $\frac{K'_O}{K'_R}$. Henderson, however, attached to this figure only the accuracy of a rough approximation to the order of magnitude. It remains to obtain precise experimental data on human hemoglobin in order to ascertain whether human and horse hemoglobin differ markedly in the properties indicated by the $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ curves.

That introduction of oxygen into the molecule of an organic acid increases the dissociation constant (reduced pK') seems to be the usual rule, although with exceptions. Table XXI, taken from Landolt-Börnstein's "Tabellen," indicates the magnitude of the change caused by introduction of oxygen into organic acids of varying types. Whether any of the acids in Table XXI are structurally related to the part of the hemoglobin molecule that binds labile oxygen is, of course, unknown. The data in the table show, however, that a pK' shift of 1.46 as the result of the introduction of oxygen in the neighborhood of an acid radicle is within the order of magnitude of the effects observed in organic acids of known structure.

While a shift of 1.46 in the pK' value of one monovalent acid group affords a sufficient and reasonable explanation of the observed facts, the possibility is not excluded that the summated effects of smaller shifts in the pK' values of more than one group may be responsible. One can demonstrate with curves such as those in Fig. 17 that identical $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$ values would result from shifts in the dissociation constants of 2 acid hydrogens such that $\text{pK}'_{R_1} - \text{pK}'_{O_1} = 1.46$ if the constants are so related that $\text{pK}'_{R_1} = \text{pK}'_{O_2}$. Similarly, if three acid dissociation constants were assumed to be reduced by deoxygenation of oxyhemoglobin, the most acid one having pK'_1 , the next pK'_2 , the same $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$

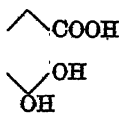
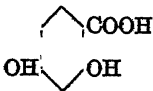
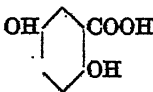
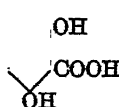
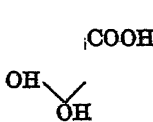
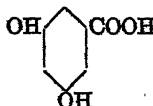
TABLE XXI.

Comparison of the Dissociation Constants of Certain Organic Acids.

Acid.	Formula.	pK	ΔpK^*
Acetic.	CH_3COOH	4.75	
Glycollic.	$CH_2OHCOOH$	3.82	0.93
Glyoxalic.	$CHOCOOH$	3.80	1.45
Propionic.	CH_3CH_2COOH	4.85	
β -Hydroxypropionic.	CH_2OHCH_2COOH	4.51	0.34
Lactic.	$CH_3CHOHCOOH$	3.85	1.00
Butyric.	$CH_3(CH_2)_2COOH$	4.82	
β -Hydroxybutyric.	$CH_2OH(CH_2)_2COOH$	4.72	0.10
Isooxybutyric.	$(CH_3)_2CHOHCOOH$	3.97	0.95
Valerianic.	$CH_3(CH_2)_3COOH$	4.8	
β -Hydroxyvalerianic.	$CH_3CHOH(CH_2)_2COOH$	4.7	0.10
Malonic.	$COOHCH_2COOH$	2.8	
Hydroxymalonic.	$COOHCHOHCOOH$	2.3	0.5
Succinic.	$COOH(CH_2)_2COOH$	4.18	
Tartaric.	$COOH(CHOH)_2COOH$	3.01	1.17
Malic.	$COOHCH_2CHOHCOOH$	3.40	0.78
Maleic.	$COOH(CH=CH)COOH$	1.89	
Dihydroxymaleic.	$COOH(COH=COH)COOH$	1.15	0.74
Benzoic.	 COOH	4.19	
<i>o</i> -Hydroxybenzoic.	 COOH OH	3.0	1.19
<i>m</i> -Hydroxybenzoic.	 COOH OH	4.08	0.11
<i>p</i> -Hydroxybenzoic.	 COOH OH	4.55	-0.36

* ΔpK is obtained by subtracting the pK of the hydroxy substituted acid from the pK of the corresponding unsubstituted acid.

TABLE XXI—*Concluded.*

Acid.	Formula.	pK	ΔpK
Dihydroxybenzoic. 1, 3, 4		2.96	1.23
1, 2, 4		3.29	0.90
1, 2, 5		2.96	1.23
1, 2, 6		1.30	2.89
1, 3, 4		4.48	-0.29
1, 3, 5		4.04	0.15

pH curve would be obtained under the conditions that $pK'_{R_2} - pK'_{O_1} = 1.46$, $pK'_{R_1} = pK'_{O_2}$ and $pK'_{R_2} = pK'_{O_3}$. On the other hand, as will be shown later in a discussion of Hill's hypothesis, an assumption that there is less than 1 labile acid hydrogen per molecule of oxygen capacity leads to conclusions incompatible with our results.

Calculation of the Base Bound by Hemoglobin from the Oxygen Saturation and the pH.

It is now possible to formulate more accurately than in our third paper (9) the equation which expresses the titration curve

of hemoglobin in the oxygenated, reduced, or inactive form. In a mixture of reduced, oxygenated, and inactive hemoglobin the total base bound by the hemoglobin is given by the expression

$$(15) \quad [\text{BHb}] = [\text{BHb}_R] + [\text{BHbO}_2] + [\text{BHbI}]$$

where BHb_i indicates the base bound to hemoglobin that is in the form inactivated with respect to oxygen-binding power.

It has been shown that the titration curve for reduced hemoglobin from the isoelectric point I_R , at pH 6.81, to pH 7.6 closely approximates a straight line. Hence

$$(16) \quad [\text{BHb}_R] = \beta_R [\text{Hb}_R] (\text{pH} - I_R)$$

Furthermore, it will be shown¹ that inactive horse hemoglobin has the same linear titration curve as reduced hemoglobin. Hence

$$(17) \quad [\text{BHbI}] = \beta_R [\text{BbI}] (\text{pH} - I_R)$$

The base bound by oxygenated hemoglobin may accordingly be expressed as that which would be bound by the hemoglobin in the reduced condition plus the amount, $\frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$, added isohydrionically as the result of oxygenation. Hence

$$(18) \quad [\text{BHbO}_2] = [\text{HbO}_2] \beta_R (\text{pH} - I_R) + \frac{\Delta[\text{BHb}]}{\Delta[\text{O}_2\text{Hb}]}$$

If we substitute in Equation 15 the values for $[\text{BHb}_R]$, $[\text{BHbI}]$, and $[\text{BHbO}_2]$ from Equations 8, 16, 17, and 18, we obtain

$$(19) \quad [\text{BHb}] = \beta_R ([\text{Hb}_R] + [\text{HbI}] + [\text{HbO}_2]) (\text{pH} - I_R) + [\text{HbO}_2] \left(\frac{1}{1 + 10^{\text{pK}'_O - \text{pH}}} - \frac{1}{1 + 10^{\text{pK}'_R - \text{pH}}} \right)$$

Since

$$[\text{Hb}_R] + [\text{HbI}] + [\text{HbO}_2] = [\text{Hb}],$$

Equation 19 simplifies to

$$(20) \quad [\text{BHb}] = \beta_R [\text{Hb}] (\text{pH} - I_R) + [\text{HbO}_2] \left(\frac{1}{1 + 10^{\text{pK}'_O - \text{pH}}} - \frac{1}{1 + 10^{\text{pK}'_R - \text{pH}}} \right)$$

The accuracy with which these equations express the titration curves is illustrated in Table XVI where a comparison is made between the values of $\frac{BHb_R}{Hb_R}$ and $\frac{BHbO_2}{HbO_2}$ obtained experimentally and calculated from Equation 20 with the numerical constants of Table XXIII.

Hill's Hypothesis Concerning the Relationship between Oxygenation and Base-Binding Power of Hemoglobin.

A modification of Henderson's hypothesis has lately been advanced by A. V. Hill (13, 14, 15), based on the assumption that, instead of 1 acid group per mol, only 1 acid group per n mols of oxygen capacity in hemoglobin has its acidity affected by oxygenation, where n has the same value as in Hill's well known oxygen dissociation equation, $\frac{1}{K} = \frac{[Hb_R] \times [PO_2]^n}{[HbO_2]}$. Ac-

cording to Hill's hypothesis n molecules of hemoglobin, capable of combining with 1 molecule each of oxygen, are combined or aggregated in some manner, and have among them only 1 labile acid hydrogen of which the dissociation constant is greater in the oxygenated than in the reduced hemoglobin. The dissociation constant of this $\frac{1}{n}$ acid group per mol of oxygen capacity is supposed to be so greatly changed by oxygenation and reduction, that in the oxygenated hemoglobin it binds at physiological pH an entire equivalent of base, in reduced hemoglobin none at all, the reaction $H(Hb)_n + n O_2 + BHCO_3 = B(HbO_2)_n + H_2CO_3$ going with practical completeness from left to right when reduced blood is oxygenated.

If Hill's hypothesis were correct, the $\frac{d[BHb]}{d[O_2Hb]}$ value at varying pH would be represented by Equation 8 with Hill's n introduced as follows:

$$(21) \quad \frac{d[BHb]}{d[O_2Hb]} = \frac{1}{1 + 10^{pK'_O - pH}} - \frac{1}{1 + 10^{pK'_R - pH}}$$

We have found it impossible to express our results by such an equation. The value of Hill's n for horse hemoglobin in

solutions at constant pH with 30 mm cation concentration is found from previously published data (9) to be about 1.5. It is possible to assume a sufficiently great $pK'_O - pK'_R$ difference to yield a maximum $\frac{d[BHb]}{d[O_2Hb]}$ value, calculated by the above equation with $n = 1.5$, approximating the maximum we have observed. At other pH points, however, the calculated curve falls less rapidly than the observed, and the differences between calculated and observed values exceed the experimental error. If we assume, for example, $pK'_O = 7.6 - 2.0$, $pK'_R = 7.6 + 2.0$, $n = 1.5$, we obtain the following results.

pH	$\frac{d[BHb]}{d[O_2Hb]}$		
	Calculated by Equation 21 with $n = 1.5$.	Observed average in solutions of 30 mm cation concentration (From Table XVIII).	Calculated by Equation 8, with pK'_O and pK'_R values from Table XVIII.
7.6	0.65	0.74	0.68
7.4	0.65	0.66	0.66
7.2	0.65	0.61	0.61
7.0	0.64	0.55	0.53
6.8	0.63	0.43	0.43

We find it equally difficult to reconcile quantitatively with Hill's hypothesis the $\frac{d[BHb]}{d[O_2Hb]}$ values determined on whole blood by Van Slyke, Hastings, and Neill (16). The first three experiments in their paper were performed at approximately constant pH, of 7.23 to 7.28 and with a range of oxygen tensions such that Hill's n could be determined. The graphic logarithmic method of Brown and Hill for determining n gives in these experiments a value of 2.54. The points for blood approximating complete reduction do not agree with the others, whether because of the greater experimental difficulties in attaining complete equilibrium and in accurately determining $\frac{HbO_2}{Hb_R}$ and p_{O_2} at low oxygen tensions, or because at such tensions Hill's equation is less exact, we are not certain. In all three experiments, however, the points for oxygen tensions above 20 mm. fall almost

exactly on the straight line indicated by the equation, $\log \frac{\text{HbO}_2}{\text{Hb}_R} = 2.54 \log p_{\text{O}_2} - 3.73$; thus substantiating Hill's oxygen dissociation equation for conditions of constant pH_e and of $p_{\text{O}_2} > 20$ mm. (Fig. 19).

However, if we insert the value of 2.54 for n in Equation 21 we obtain as the *maximum possible value* of $\frac{d[\text{BHb}]}{d[\text{O}_2\text{Hb}]}$

(with $\frac{K_0}{K} \approx \infty$) $\frac{1}{2.54} = 0.39$, while the observed values were 0.55, 0.55, and 0.54 in the three experiments, and would be over 0.6 if corrected to constant pH_e instead of constant pH_s .

Our results, therefore, do not indicate that one is justified in extending the n of Hill's oxygen dissociation equation to include the significance he attributes to it in explaining the relative acidities of oxygenated and reduced hemoglobin.

Physiological Efficiency of Hemoglobin as a Carrier of Carbon Dioxide and Oxygen.

From the neutrality-regulating standpoint one may define the most efficient carrier of carbon dioxide and oxygen as the one which accomplishes their transport with minimum reaction change. It is of interest to estimate from our data how nearly horse hemoglobin approaches perfection as such a carrier.

If we consider a normal resting organism with a respiratory quotient of 0.8 and an optimum pH_e within the erythrocytes of 7.30 (see Van Slyke, Wu, and McLean (1)) the perfectly efficient carrier might be considered as one which could enable the blood to exchange 1 mol of O_2 for 0.8 mol of CO_2 without altering the pH_e from the above optimum. It is necessary as a condition that at pH_e 7.3 the addition of 1 mol of oxygen shall shift the pK' of one or more buffer groups of the substance to such an extent that 0.94×0.8 equivalent, or 0.75 equivalent, of base is set free from the carrier to combine with CO_2 as BHCO_2 (the other 0.06 mol of CO_2 is absorbed as free H_2CO_2 without change of pH , since at pH 7.3 the ratio $\text{H}_2\text{CO}_2:\text{BHCO}_2$ is approximately 6:94). With a pair of curves like those of Fig. 17 one may estimate

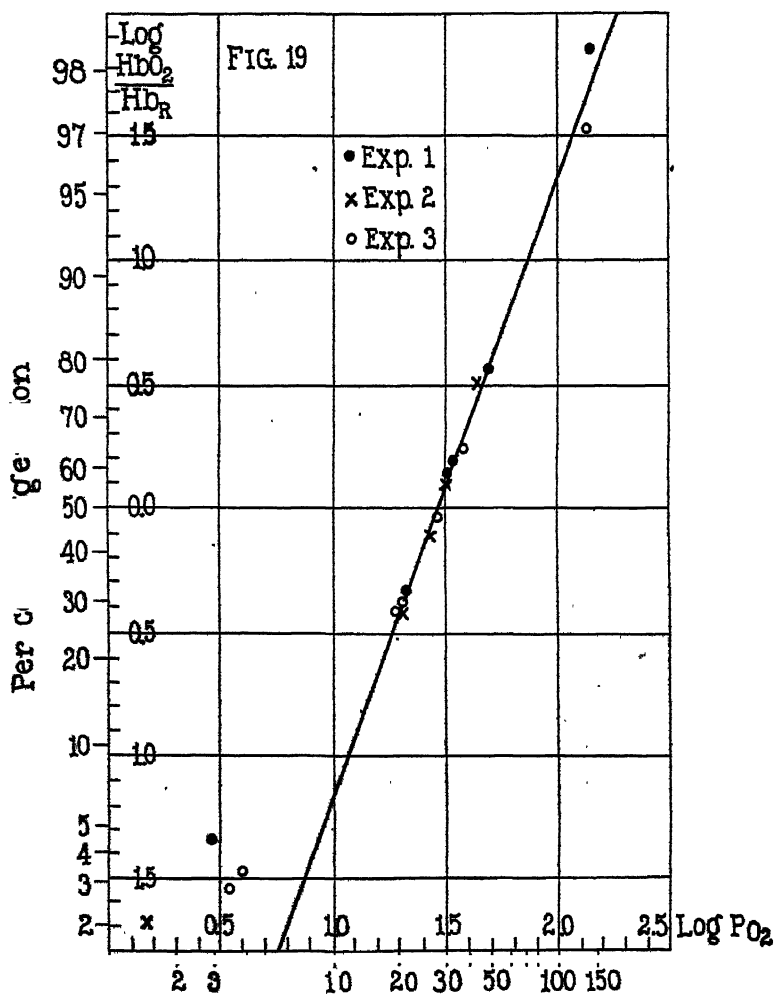


FIG. 19. Data from experiments of Van Slyke, Hastings, and Neill (16), plotted according to linear logarithmic form of Hill's equation, $\log \frac{\text{HbO}_2}{\text{Hb}_R} = n \log p_{\text{O}_2} + \log K$. The equation for the straight line is satisfied by the values $n = 2.54$ and $\log K = -3.73$.

what sort of substance fulfills these requirements. A buffer will meet them if it has one carboxyl whose pK' , when 1 mol of O_2 is absorbed, becomes shifted from $7.3 + 0.85$ to $7.3 - 0.85$, or from 8.15 to 6.45. These ideal figures approach the values $7.30 + 0.73$ and $7.30 - 0.73$, or 8.03 and 6.57 estimated from our titration curves of reduced and oxygenated hemoglobin, respectively, in the presence of 0.145 equivalent cation concentration. A buffer group shifting its pK' from 6.57 to 8.03 would free 0.68 equivalent of alkali instead of the 0.75 equivalent required for a perfectly isohydronic process.

The buffer value of hemoglobin, aside from that connected with the oxygen change, is such that the slight additional amount of alkali required to combine with the remaining 0.07 mol of CO_2 per mol of O_2 exchanged would be set free with a reaction change of about 0.01 pH.

This value is calculated as follows. The molecular buffer value of hemoglobin at H 7.3 is 2.9; viz., $\frac{\Delta[BHb]}{[Hb] \Delta pH} = 2.9$. (Our data indicate this buffer value for both reduced and oxygenated hemoglobin at 7.3.) If we assume that the oxygen exchange is equivalent to one-third the oxygen capacity, we may express it as $\Delta O_2 = 0.33 [Hb]$. The CO_2 that must be neutralized by buffers is $0.07 \Delta O_2$, or $0.023 [Hb]$. From the formula for the buffer value of Hb we have $\Delta pH = \frac{\Delta[BHb]}{2.9 [Hb]}$. If $\Delta [BHb] = 0.023$, we have $\Delta pH = \frac{0.023 [Hb]}{2.9 [Hb]} = 0.008$. This corresponds with the normal respiratory pH_e change indicated by the nomogram in Fig. 5, Paper V (1). As indicated by the above nomogram, the pH_e change would be about three times as great as the pH_e change.

Hemoglobin combines, therefore, within itself three properties: (1) ability to combine and release almost an entire molecule of oxygen at atmospheric and tissue tensions; (2) ability so to change its base-binding power with oxygenation and reduction that the base released is equivalent to a large part of the CO_2 normally exchanged for oxygen; and (3) high buffer value at physiological pH range.

Other substances, e.g. methylene blue, can act as oxygen carriers. Organic acids of known structure, as already seen in Table XXI, show decrease in pK value of magnitudes near the

decrease shown by hemoglobin, as the result of combining with oxygen. Other proteins are efficient buffers at blood pH (see Table XXII). But in possessing the three properties combined, balanced, and active within physiological gas tension and reaction ranges, hemoglobin shows unique adaptation to its function as carrier of carbon dioxide and oxygen.

TABLE XXII.

Substance.	Buffer value per gram at pH = 7.0	Authority.
	$\frac{dB}{[P]dpH}$	
Gelatin.....	0.06	Loeb.
Egg albumen.....	0.07	"
Globulin.....	0.06	Hitchcock.
Serum protein.....	0.07	Van Slyke, Wu, and McLean.
Casein.	0.20	Loeb.
Hemoglobin.	0.16	Van Slyke, Hastings, Heidelberger, and Neill.
Phosphate. . .	3.44	Clark.

SUMMARY.

Results with Horse Hemoglobin.

Isoelectric Points.—The isoelectric point, I_R , of reduced horse hemoglobin, defined as the point at which equal amounts of base and acid are bound, is at pH 6.81 ± 0.02 .

The isoelectric point, I_O , of oxyhemoglobin is somewhat lower, apparently slightly below pH 6.7. As it is more acid than the lowest pH values on our experimental curves, and can only be approximated by extrapolation, its value is less certain than that of I_R .

Buffer Values.—Between the isoelectric point and pH 7.6 the molecular buffer value, β_R , of reduced horse hemoglobin is almost constant for a given solution. In solutions of different electrolyte content the value of β_R appears to increase appreciably with the salt concentration, as shown in Table XXIII.

For oxyhemoglobin the buffer value in a given solution decreases steadily from pH 6.8 to 7.6 (see Table XVII).

Nature of the Oxidation-Reduction Effect on the Acidity of Hemoglobin.—The increase in base-binding power that occurs when reduced hemoglobin is oxygenated at varying pH follows a curve quantitatively consistent with Henderson's hypothesis that combination with a molecule of oxygen increases the dissociation

TABLE XXIII.
Numerical Values for Constants of Horse and Dog Hemoglobin.

Hemoglobin species.	Cation concentration in solution.	β_R Molecular buffer value of reduced hemoglobin.	I_R Isoelectric point of reduced hemoglobin.	pK'_O	pK'_R	$\frac{K'_O}{K'_R}$
	mm					
Horse.	30	2.6	6.81	6.87	8.33	29
"	50	2.7	6.81	6.67	8.13	29
"	145	2.9	6.81	6.57	8.03	29
Dog.	30	2.0	6.81	6.98	8.22	17

constant of 1 acid hydrogen in the hemoglobin molecule. The experimentally obtained curves are expressed by the equation, derived from the mass law, as follows:

$$\frac{d[BHb]}{d[O_2Hb]} = \frac{1}{1 + \frac{[H^+]}{K'_O}} \cdot \frac{1}{1 + \frac{[H^+]}{K'_R}}$$

$$= \frac{1}{1 + 10^{pK'_O - pH}} \cdot \frac{1}{1 + 10^{pK'_R - pH}}$$

where $\frac{d[BHb]}{d[O_2Hb]}$ is the isohydronic ratio

$\frac{\text{increase in equivalents of base bound by hemoglobin}}{\text{increase in mols of } O_2 \text{ bound by hemoglobin}};$

K_O and K_R are the acid dissociation constants, in the oxygenated and reduced states, respectively, of the 1 acid hydrogen which is assumed to have its dissociation increased by oxygenation.

$pK' = -\log \frac{K}{\gamma}$, where γ represents the dissociation of the salt.

The same results can also be calculated by assuming that oxygenation causes lesser increases in the K' values of more than

one buffer group, but there is at present no reason to prefer such an explanation.

Our $\frac{d[\text{BHb}]}{d[\text{O}_2\text{Hb}]}$ values do not follow the equation

$$\frac{d[\text{BHb}]}{d[\text{O}_2\text{Hb}]} = \frac{1}{n} \left(\frac{1}{1 + 10^{pK' - pH}} - \frac{1}{1 + 10^{pK'_R - pH}} \right)$$

required by Hill's hypothesis, that only one acid H in the aggregate hemoglobin molecule $\text{H}[\text{Hb}]_n$ has its dissociation constant affected by oxygenation and reduction.

Equation Expressing the Relationships of Base-Binding Power, Reaction, and Degree of Oxygenation of Hemoglobin.—The relationships outlined above between base-binding power, reaction, and degree of oxygenation are expressed in the equation

$$[\text{BHb}] = \underbrace{\beta_R [\text{Hb}] (pH - I_R)}_{\text{Base bound by hemoglobin in the reduced state.}} + \underbrace{[\text{HbO}_2] \left(\frac{1}{1 + 10^{pK'_O - pH}} - \frac{1}{1 + 10^{pK'_R - pH}} \right)}_{\text{Additional base bound as result of oxygenation.}}$$

($[\text{Hb}]$ indicates total hemoglobin in mols of oxygen capacity, $[\text{HbO}_2]$ the oxyhemoglobin.) This equation expresses the observed relationships more accurately than the approximate linear equation of Van Slyke, Hastings, Heidelberger, and Neill (10). The values of the constants of the equation are given in Table XXIII.

Results with Dog Hemoglobin.

The relationships with dog hemoglobin are similar to those in horse hemoglobin, but the numerical constants, particularly the β_R and I_R values, are unmistakably different, as may be seen from Table XXIII. The differences in the values for dog as compared with horse hemoglobin constitute definite and measurable chemical differences between the hemoglobins from the two species. They support the conclusion reached by Reichert and Brown from crystallographic studies, that hemoglobins from different species are different substances.

We acknowledge the assistance of Mr. John Plazin and Mr. Julius Sendroy, Jr., who performed a considerable part of the quantitative determinations on which this paper is based.

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ON THE CHEMISTRY OF THE PYRIMIDINES.

VI. NEW COLOR TESTS FOR URACIL AND CYTOSINE.

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(Received for publication, March 26, 1924.)

The study of the action of ferrous bicarbonate plus air on the pyrimidines¹ has been continued and the chemical change in the pyrimidine ring examined in more detail.

If an aqueous solution of cytosine is treated with the system ferrous sulfate plus sodium bicarbonate plus air, hydrolysis of the amino group occurs with the formation of uracil and the liberation of ammonia. Since uracil is formed from cytosine it might be assumed that in the further reaction of the system ferrous bicarbonate plus air, identical results should be expected, whether cytosine or uracil were used as the starting material. That is, however, not the case because the ammonia released from cytosine influences the reaction. The subsequent course of this influence will be described later in this paper.

Before the mechanism of the chemical changes in the pyrimidines, uracil and cytosine, was cleared up, it seemed as if uracil and cytosine behaved entirely differently, since different but characteristic color reactions were obtained in each case.

In our previous experiments with uracil and cytosine, we had studied the behavior of these compounds towards sodium-pentacyano-aquo ferroate (aquo salt) and had found that the two pyrimidines form penetration compounds of different colors.

¹ Johnson, T. B., and Baudisch, O., *J. Am. Chem. Soc.*, 1921, xliii, 2670. Baudisch, O., and Johnson, T. B., *Ber. chem. Ges.*, 1922, lv, 18. Deuel, H. J., and Baudisch, O., *J. Am. Chem. Soc.*, 1922, xlv, 1581. Pfaltz, M. H., and Baudisch, O., *J. Am. Chem. Soc.*, 1923, xlv, 2972. Bass, L. W., and Baudisch, O., *J. Am. Chem. Soc.*, 1924, xlvi, 181. Baudisch, O., and Bass, L. W., *J. Am. Chem. Soc.*, 1924, xlvi, 184. Bass, L. W., *J. Am. Chem. Soc.*, 1924, xlvi, 190.

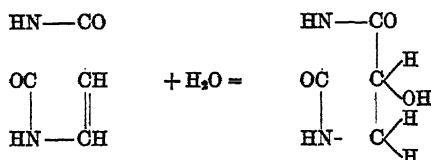
With the salt mentioned, uracil forms a deep green, and cytosine, a red compound. This is due to the fact that in uracil the ethylene group with its partial valences is linked to the central iron atom. Ethylene itself forms with the aquo salt a deep green compound. In cytosine not the ethylene group but the nitrogen of the amino group determines the character of the compound, just as in indole. Due to the nitrogen of the pyrrole ring of indole, a red penetration compound is formed and not a green one. This experimental result must be emphasized here, since it apparently shows, very directly, the affinity of pyrimidines for ferrous salts—a fact which is of great importance for further study with pyrimidines. Another result of our previous investigations on uracil and cytosine is the fact that after treatment with ferrous bicarbonate plus air, the ring of the pyrimidines is still intact, but after warming on the water bath, hydrolysis takes place with the formation of urea. Nothing could be said at that time regarding the fate of the remaining three carbons of the ring, so the experiments were continued with a view to obtaining a deeper insight into the chemical changes involved in the disruption of the pyrimidine ring.

The present paper describes the results of a new observation which should be emphasized at this point before passing on to a more detailed description. Uracil and cytosine form on treatment with ferrous bicarbonate and air and subsequent autoxidation of the colorless intermediate products, the same brilliant lemon-yellow pigment possessing special chemical properties. The formation of this pigment from the colorless intermediate compounds by such mild treatment is not only quite remarkable from biological, but also from a physical standpoint, since by this change the absorption of light is extended far into the visible spectrum.*

It should also be strongly emphasized that the pyrimidines, uracil and cytosine, which are stable in a weakly alkaline solution, become, on treatment with ferrous bicarbonate and air, autoxidizable systems which form a number of biochemically important compounds on successive absorption of oxygen.

* It is worth mentioning that fresh urine, the color of which resembles the aforementioned yellow pigment, also gives all the characteristic reactions, so that one may suspect that the new compound may be contained in urine.

The system ferrous sulfate plus sodium bicarbonate plus air does not, as one would expect, oxidize the pyrimidines, but causes the addition of 1 molecule of water to the ethylene group.



The same compound is formed from uracil and cytosine, namely dihydro-isobarbituric acid, which, up to the present time, could not be isolated, but its formation is indicated by several characteristic chemical reactions.

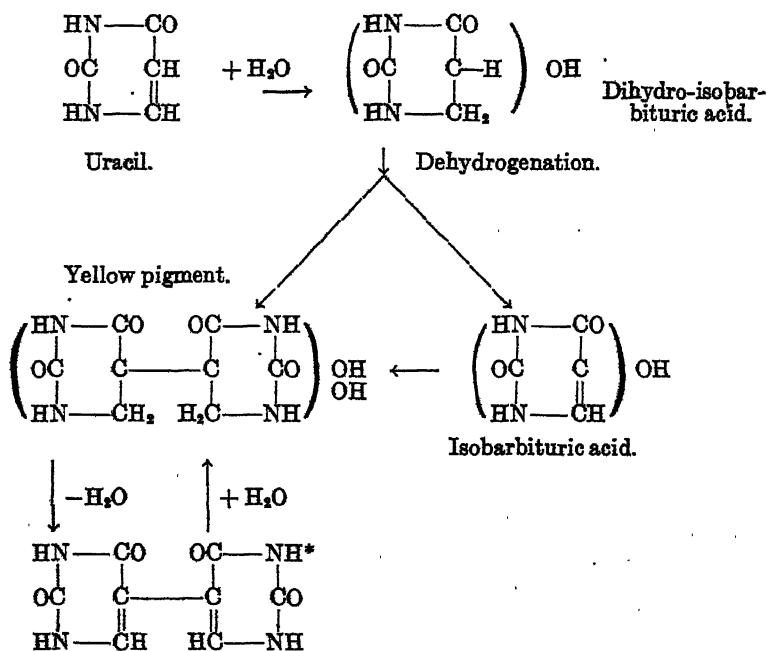
After treatment of an aqueous cytosine or uracil solution with ferrous bicarbonate and air and filtration of the red ferric hydroxide, a colorless, strongly autoxidizable solution is obtained. This solution is then gradually converted by the further absorption of oxygen from the air into the deep red complex ferrous salt of isobarbituric acid. On further autoxidation the deep red solution changes color from the surface downward into brilliant yellow. In alkaline solution at 37°C. this yellow pigment is weakly autoxidizable itself—a fact which is illustrated by the gradual disappearance of the yellow color from the surface.

If one removes the iron of the deep red complex ferrous salt obtained from cytosine after treatment with ferrous bicarbonate and air and subsequent autoxidation, by the addition of nitroso-phenyl-hydroxylamine-ammonium (Cupferron)³ in a weakly acid solution in the cold, a brilliant yellow solution of the new compound is also obtained which is free from iron.

The same yellow compound is formed from a synthesized isobarbituric acid if the aqueous solution of the acid, together with small amounts of ferrous sulfate and bicarbonate, is allowed to stand in an open dish for 2 or 3 days. After addition of *m*-phosphoric acid and evaporation to dryness in the water bath, a brilliant yellow residue remains, which shows all the characteristic properties of the yellow compound obtained from uracil or cyto-

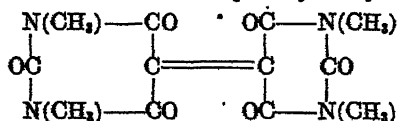
³ Baudisch, O., *Chem. Ztg.*, 1909, xxxiii, 1298. Baudisch, O., and King, V. L., *J. Ind. and Eng. Chem.*, 1911, iii, 629.

sine. The question now arises as to the chemical constitution of the new yellow compound. Although it has not been possible to analyze the yellow pigment because of the small quantities of pyrimidine which were at our disposal, it seems probable that its formation occurs in the following way.

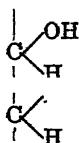


According to this scheme it will be seen that we are dealing with the formation of a bimolecular pinacone-like compound, with the linking of two carbon atoms, 5 of the pyrimidine ring, a property which is characteristic of the atomic group.

*This compound resembles tetra methyl-dehydro-hydurilic-acid



which was first prepared by Biltz and Hamburger (Biltz, H., and Hamburger, T., *Ber chem. Ges.*, 1916, xlix, 655).



This type of reaction, as we shall see later, plays an important rôle in biochemical oxidation-reduction processes and in biochemical processes in general. As concerns the experimental part of the conversion of cytosine or uracil by means of ferrous bicarbonate and air, it was very apparent that the yellow pigment was formed much more quickly from cytosine than from uracil. This was found to be due to the fact that the ammonia produced in the cytosine experiment acts as a catalyst and hastens the autoxidation and, therefore, the formation of the yellow pigment. This pigment, which is characterized by a great stability toward both acids and alkalis, has special properties which will now be described in more detail.

The chemical reactions of the yellow pigment are quite different according to whether one deals with weakly acid or weakly basic solution. In basic solution, even at a very high dilution, the following characteristic reactions were observed. Ammoniacal silver nitrate solution is slowly reduced in the cold and is reduced very quickly on heating. The reducing power is very marked towards phosphomolybdic acid with the formation of a deep blue color. Especially sensitive is the reaction with diazobenzenesulfonic acid, which gives an intense, but temporary, brilliant red color. When the solution is cooled with ice before adding the diazo compound, the red color can be maintained for a considerable time.

The reaction with methylene blue is also very characteristic. Methylene blue solutions are decolorized quickly but an intense blue color appears at the surface due to the absorption of oxygen from the air. This reaction characterizes the reduction and oxidation power of the yellow pigment in alkaline solutions. Methylene blue is reduced to the leuco compound because of the loosely linked hydrogen atoms, while on the surface of the liquid a peroxide is formed by the absorption of oxygen which afterwards decomposes with the formation of hydrogen peroxide. Such oxidizing and reducing systems are particularly important in biochemical

processes, and groups which bring about such reactions appear in very different aliphatic and aromatic compounds in nature. In acid solution, the yellow pigment is very susceptible to reducing agents. For instance, zinc dust easily decolorizes it. On warming the colorless solution in an open bath, the yellow color returns on autoxidation.

These new experimental results in the study of the pyrimidines, uracil and cytosine, give, I believe, quite a new insight into the part which they play in biochemical processes—a field in which we have heretofore been completely in the dark.

Significance of the Formation of a Yellow Pigment from Cytosine and Uracil for Biochemistry.

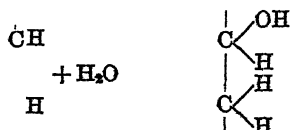
Among the decomposition products of uric acid we find a number of pyrimidine combinations which have aroused the attention of biochemists since the early days of Liebig and Wöhler. The simple pyrimidines, uracil, cytosine, and thymine, however, have not been given sufficient attention by the biologist or the biochemist, and, therefore, we know practically nothing of the metabolism of these compounds in the plant or animal organism.

Prior to the investigations of Kossel and of Levene and Jacobs, it was still an unsolved question whether the pyrimidines appear as such in the nucleic acids or whether they are decomposition products of the purines. As a result of the investigations of Levene and Jacobs⁴ we now know that there are simple combinations of pyrimidines with sugar and phosphoric acid, so called nucleotides, which may be considered as the real components of nucleic acid. Still unsolved remained the biochemistry of the pyrimidine ring itself and nothing was known about the chemical properties of uracil, cytosine, and thymine as regards their relation to other compounds occurring in the plant or animal kingdom. In the following description will be given an outline of such relationships which are based on experimental observations, with instances such as indole, chrysarobin, quercetin, and adrenalin.

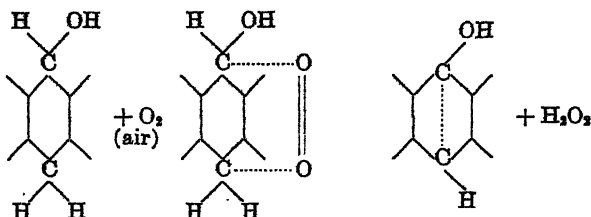
In the pyrimidine ring we have a very reactive group, namely the ethylene group, which we find also in indole and somewhat

⁴Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1908, xli, 2703; 1909, xlii, 335, 1198, 2469, 2474; 1911, xliv, 746; *Handbuch der biologischen Arbeitsmethoden*, Berlin, 1910, ii, 605.

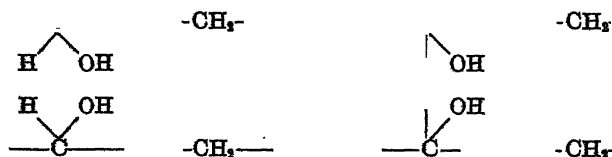
masked in anthracene. On addition of 1 water molecule a new atomic configuration is obtained



of a very reactive type and of special biochemical significance. Three reactions are particularly characteristic of this group as we shall now see. First, in alkaline solution this atomic configuration absorbs oxygen from the air with the formation of an intermediate peroxide which itself is unstable and decomposes with the production of hydrogen peroxide.

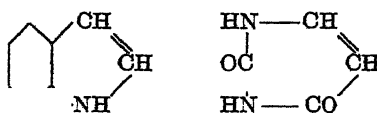


Second, 2 hydrogen atoms of the group in the presence of OH ions are very reactive and readily reduce to the leuco compounds such substances as methylene blue or indigo carmine. Third, one of the carbon atoms of the group possesses the power of linking other carbon atoms which results, of course, in the formation of bimolecular compounds according to the following scheme.



Such a bimolecular compound is obtained, for instance, with adrenalin and a solution of this substance gives all the previously described color reactions which are characteristic for an aqueous solution of isobarbituric acid or of the yellow compound ob-

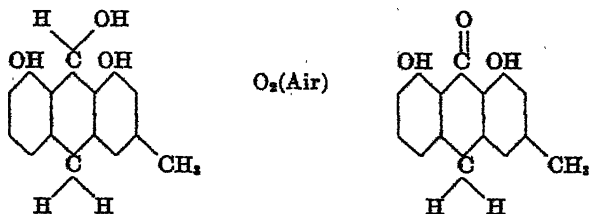
tained from cytosine and uracil. If we now compare indole and uracil



we will find that because of the ethylene groups in the ring many chemical properties of the two substances are alike. For instance, on treatment of indole with ferrous bicarbonate and air, a colorless solution is first obtained which gives the same characteristic reactions observed after treatment of uracil or cytosine with the same reagent, as for instance in its behavior towards such reagents as ammoniacal silver nitrate, phosphomolybdic acid, diazobenzene-sulfonic acid, and the like.

The colorless solution obtained after shaking indole, with the system ferrous sulfate plus sodium bicarbonate and air is strongly autoxidizable, and due to the absorption of oxygen from the air becomes greenish yellow and finally indigo is formed.

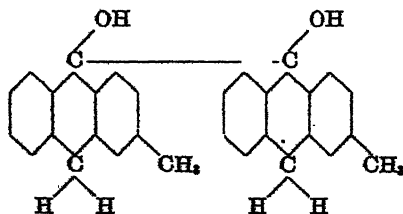
A similar behavior is found among anthracene compounds which are widely distributed in nature. For instance, chrysarobin, a well known remedy for skin diseases,⁵ which forms on reduction dihydroanthranol



1,8-Dihydroxymethyl dihydroanthranol.

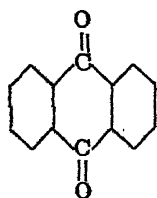
Chrysarobin.

and as a by-product anthrapinacone.

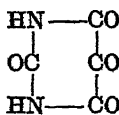


It is quite interesting to compare all the steps involved in the transformations of anthracene to anthraquinone and of uracil to alloxan. All the compounds listed in the following table have been isolated and are described in the literature except dihydro-isobarbituric acid which is first mentioned in this paper.

	Anthracene.		Uracil.
	Dihydroanthracene.		Dihydro-uracil.
	Dihydroanthranol.		Dihydro-isobarbituric acid.
	Anthranol.		Isobarbituric acid.
	Oxanthrol.		Isodialuric acid.

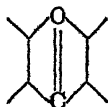


Anthraquinone.

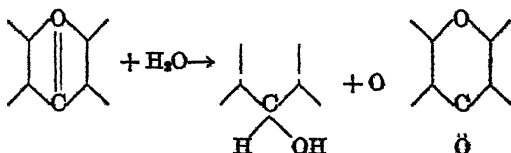


Alloxan.

Quite similar to the behavior of the anthracene derivatives concerning the attachment of 1 water molecule to a double carbon linkage, is the behavior of the group



which occurs in the anthocyanins. The formation of a cyanidine pseudo base by the addition of a water molecule to the carbon atom and finally oxidation to a flavone compound



resembles very much the aforementioned synthesis of dihydroisobarbituric acid from uracil. Also, in this case we have to deal with a system which is stable in acid solution and which becomes very reactive and autoxidizable in weak alkaline solution. It must be emphasized that in the natural pigments mentioned as well as in the pyrimidines, we have to do mostly with the glucosides of compounds which, according to our knowledge of certain anthocyanins, have a stronger reactivity than the compounds themselves.

If we summarize our observations we can assume that the pyrimidines, uracil and cytosine, or their decomposition products dihydrobarbituric acid, isobarbituric acid, and the brilliant yellow compound, possess the functions of respiration pigments. This explanation at the same time gives a deeper insight into the necessity of iron which, as is well known, can be found in every cell nucleus.

EXPERIMENTAL WORK.

Experiment 1.—On adding a solution of 50 gm. of ferrous sulfate in 100 cc. of distilled water to a solution of 50 gm. of sodium bicarbonate and 0.1 gm. of uracil in 1 liter of water, a white precipitate of ferrous bicarbonate was formed which gradually turned red on shaking with air. After filtration of the ferric hydroxide a clear, colorless solution remained which, on being allowed to evaporate in vacuum to about 150 cc., yielded a liquid which was clear and colorless (Solution S) but which immediately turned reddish upon contact with air. The reddish solution gave the following characteristic reactions.

With ammoniacal silver nitrate, reduction took place even in the cold; with phosphomolybdic acid an indigo blue color was formed. With diazobenzenesulfonic acid a brilliant red color, quickly fading to yellow, was produced. On acidifying with *m*-phosphoric acid and evaporating to dryness in the water bath, there remained a brilliant lemon-yellow residue. But on acidifying with glacial acetic acid a residue remained which had an eosin-like color.

All these reactions are given only with a uracil solution that had been treated with ferrous bicarbonate and not with the uracil solution itself.

Another striking color reaction is the gradual change from colorless into red, then brilliant yellow, and again colorless, which took place if one allowed Solution S to stand in the incubator. The color change always begins at the surface, which indicated that the chemical process was due to an autoxidation process.

Experiment 2.—This experiment was an exact repetition of the first one, with the exception only that the mixture was not shaken by hand but stirred with a very good stirrer for several hours. After filtration and evaporation in vacuum to dryness the white residue became eosin red on contact with the air. While in the first experiment the reddish color was due to the formation of a complex ferrous salt of isobarbituric acid, here we had to do with an organic compound of red color which, however, gave all the same reactions described above.

Experiment 3.—In this experiment, cytosine was taken instead of uracil. The conditions were exactly the same as in Experiment 1 and the shaking was done by hand for 1 hour. Also, in this case the remaining solution after vacuum distillation was colorless, but changed to a deeper red than in the case of the uracil experiment. All the above mentioned characteristic reactions were observed, with the single exception that in the phosphomolybdic acid reaction, the color was greenish blue instead of indigo blue. This is due to the fact that in the cytosine experiment the yellow pigment was more rapidly formed than in the case of uracil and the mixture of blue and yellow gave the more green appearance.

Experiment 4.—The working conditions were changed as follows: In a 2 liter Pyrex round bottom flask $1\frac{1}{2}$ liters of distilled water were boiled for 1 hour and then 0.1 gm. of uracil and 10 gm. of NaHCO_3 were added. After this, a test-tube was filled with 10 gm. of powdered ferrous sulfate

and suspended in the neck of the bottle. After further boiling for about 15 minutes, the bottle was closed air-tight, quickly cooled by tap water to room temperature, and then, by turning the flask, the ferrous sulfate mixed with the sodium bicarbonate was emptied out of the test-tube into the solution. The precipitated snow-white ferrous bicarbonate takes up oxygen rapidly and after vigorous shaking with air, all the iron had changed in about 20 minutes into the red ferric hydroxide. After filtration and evaporation to dryness in vacuum, a colorless residue remains, which, after solution in water, becomes reddish. This solution gave all the aforementioned reactions.

Experiment 5.—This experiment was similar to Experiment 4, except that cytosine was used. In this case the white ferrous bicarbonate did not change so quickly into the red ferric hydroxide because of the ammonia split off from the cytosine by hydrolysis. A dark blue-green precipitate was formed, which changed after further shaking with air into the red ferric salt. The residue from the vacuum distillation was again colorless but still could be changed with air into a reddish or eosin red color. By dissolving in water, the color changes only to yellow. This liquid also gave all the characteristic reactions.

*Sensitiveness of the New Color Reactions for Uracil and Cytosine.**

A solution of 0.01 gm. of uracil or cytosine in 1 liter of water was boiled for half an hour and then 5gm. of sodium bicarbonate were added.

The ferrous sulfate was put in a test-tube and not allowed to be mixed with the boiling liquid. After 10 minutes boiling the flask was closed air-tight and suddenly cooled by tap water. After mixing the ferrous sulfate with the liquid, white ferrous bicarbonate was precipitated and it took only about 20 minutes to change it into red ferric hydroxide. After filtration and evaporation *in vacuo*, there resulted a colorless residue which was dissolved in 30 cc. of water. The solution changed gradually to a yellowish red color. For the different reactions only 1 cc. of this liquid was taken for each one. The reactions were as follows:

With ammoniacal silver nitrate solution on warming—intensive reduction. With diazobenzene sulfonic acid—a brilliant red color which quickly faded. With phosphomolybdic acid—a deep blue color.

This experiment proves the very great sensitiveness of the new color reactions for uracil and cytosine.

Experiment 6.—Experiment 5 was repeated, but the precipitated ferrous bicarbonate was allowed to stand in the absence of air for about 3 hours before it was shaken with air. The aged ferrous hydroxide absorbed the oxygen much more slowly and after 1 hour of vigorous shaking, the iron precipitate was only greenish brown and not red. After filtration and evaporation in vacuum, the residue did not give any of the characteristic reactions.

* See Wheeler, H. L., and Johnson, T. B., *J. Biol. Chem.*, 1907, iii, 183.

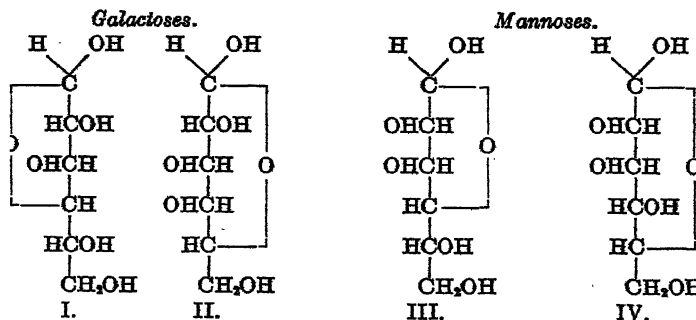
TWO ISOMERIC TETRAMETHYL MANNONOLACTONES.

BY P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 17, 1924.)

It was pointed out in previous communications that mannose in many respects behaved abnormally. The causes of its abnormal conduct have not yet been explained entirely satisfactorily. The possibility for abnormal conduct which to-day first comes to the front is the nature of the oxidic ring. True, for the present, there exists little experimental evidence to connect the structure of the ring with the optical rotation of the sugar. Only for the pentacetates of galactose, four isomers, or rather two pairs of two isomers, were described. The magnitude of the rotation of carbon atom 1 was found practically the same for each pair of isomers. There was found, however, a difference in the behavior of the two forms. In one pair the catalytic effect of zinc chloride was normal. It converts the levo-rotatory form into the dextro with much higher velocity than it reverses the dextro-rotatory form. In the other pair, the phenomenon was reversed; namely, the conversion of the dextro-rotatory isomer proceeding with greater velocity than the reverse reaction. Thus, it is not excluded that in the second pair the β -isomer is the dextro-rotatory form. In the case of galactoses, such an assumption would not be entirely unjustified for the reason that on comparing the butylene (I) and the amylenes (II) oxidic forms, one notices that the oxygen rings in the two forms have opposite directions.



It is possible then that the α - and β - isomers in sugars with different oxidic structures are determined by the position of the hydroxyls not with respect to the hydroxyls on the other carbon atoms, but by its position with respect to the oxidic ring. In the case of mannose, both the butylene oxidic and amylenic oxidic rings have the same direction and it is not excluded that some of the abnormalities might be due to a peculiarity in the oxidic ring of mannose. Of course it is not excluded that in solution the two oxidic forms reach an equilibrium. In view of these considerations, it was concluded to compare the lactones obtained on oxidation of tetramethyl mannose and that obtained on methylation of mannonic lactone. Pryde¹ has shown that in the case of galactonic lactone, the two differed in the direction of their optical rotation. On the basis of the rule of Hudson, which connects the direction of rotation of sugar lactones with the position of the lactonic ring, the 1, 5-lactone should rotate to the right and the 1, 4 to the left. In the case of the mannoses, this was not to be expected, but it was hoped to differentiate the two lactones if two isomers were formed by reducing the two lactones to the corresponding sugars. Fortunately, this step was not necessary for the reason that the two lactones showed unmistakable differences. The tetramethyl lactone obtained from the tetramethyl mannose is a liquid. The free acid and its sodium salt are dextro-rotatory; also, the lactone is dextro-rotatory. The tetramethyl lactone obtained on methylation of mannonic lactone is crystalline, and has a sharp melting point at 107°C. The free acid and its sodium salt are levo-rotatory. The lactone is dextro-rotatory. It is peculiar that the two tetramethyl lactones should rotate in opposite directions. This peculiarity requires elucidation.

Considerable interest is also attached to the fact that in the non-methylated acids the free acids and their salts rotate in opposite directions. On the other hand, the direction of rotation remains the same in the acids and the salts of the methylated derivatives.

The work of reducing the lactones to the corresponding sugars is now in progress.

¹ Pryde, J., *J. Chem. Soc.*, 1923, cxxiii, 1898.

EXPERIMENTAL.

Tetramethyl Mannonolactone.—Mannonolactone was methylated in portions of 20 gm. by the silver oxide-methyl iodide method. For the first methylation, methyl alcohol was used as solvent; for the second, acetone. For the third and final methylation, no extra solvent aside from methyl iodide was used. The combined products of several methylations were extracted with ether, dried with anhydrous sodium sulfate, the ether was removed under diminished pressure, and the syrup distilled. The larger part boiled at 135°C. at 0.3 mm. pressure and crystallized on standing at room temperature. The substance was readily recrystallized from ether and obtained as white shining crystals, melting at 107°C.

The analytical values showed this substance to be tetramethyl mannonolactone.

0.0992 gm. substance: 0.1862 gm. CO₂ and 0.0696 gm. H₂O.

0.1022 " " 0.4096 " Ag I (Zeisel).

C₁₆H₁₈O₆. Calculated. C 51.20, H 7.70, CH₃O 53.0.

Found. " 51.19, " 7.80, " 52.9.

0.1077 gm. of substance was dissolved in an excess (10 cc.) of 0.1 N NaOH, allowed to stand at 50° for several hours, and titrated with 0.1 N HCl. 4.8 cc. of 0.1 N NaOH were neutralized by the substance, equivalent to a molecular weight of 225. Calculated for the lactone the molecular weight = 234.

The lactone showed the following optical rotations.

In Water.

Initial.	Equilibrium.
$[\alpha]_D^{25} = \frac{+1.25^\circ \times 100}{1 \times 1.916} + 65.2^\circ$	$[\alpha]_D^{25} = \frac{+1.08^\circ \times 100}{1 \times 1.916} + 56.3^\circ$

In 0.3 N HCl.

Initial.	Equilibrium.
$[\alpha]_D^{25} = \frac{+1.10^\circ \times 100}{1 \times 1.770} = +62.1^\circ$	$[\alpha]_D^{25} = \frac{+0.70^\circ \times 100}{1 \times 1.770} + 39.5^\circ$

The Optical Rotation of the Free Acid.

The substance was dissolved in 5 cc. of 0.4 N NaOH, allowed to stand at about 50° for 2 hours, then cooled and acidified with 5 cc. of 0.6 N HCl. After the initial reading, the solution was kept at 50°C. until equilibrium was attained.

170 Isomeric Tetramethyl Mannonolactones

Initial.	Equilibrium.
$[\alpha]_D^{25} = \frac{1}{1 \times 1.974} = -25.3^\circ$	$[\alpha]_D^{25} = \frac{+0.95^\circ \times 100}{1 \times 1.974} = -48.2^\circ$

The Optical Rotation of the Sodium Salt.

The lactone was dissolved in 0.2 N NaOH and warmed at 50° for 2 hours.

$$[\alpha]_D^{25} = \frac{-0.42^\circ \times 100}{1 \times 1.856} = -22.5^\circ$$

Tetramethyl Mannose.—This was prepared by the methylation of methyl mannoside with dimethyl sulfate and sodium hydroxide as described by Haworth. The mixture of methylated mannosides obtained by this process was converted completely into tetramethyl mannoside by the sodium-methyl iodide process of Freudenberg and Hixon.²

The product obtained in this way is distilled at 135–139°, $p = 1$ mm. The distillate crystallized nearly at once on reaching room temperature. The tetramethyl mannoside is converted into the free sugar by hydrolysis with 8 per cent hydrochloric acid as described by Irvine and Moody.³

Oxidation of Tetramethyl Mannose.—50 gm. of tetramethyl mannose were dissolved in 250 cc. of water and 2 parts of bromine added gradually, all being kept at room temperature for 2 days. The bromine was removed in the usual way and the solution was concentrated under diminished pressure to a very thick syrup. This was extracted with dry ether. The ethereal solution was again dried over sodium sulfate, concentrated, and distilled.

The syrup was fractionated as follows:

Fraction I boiled at 110–115°, $p = 0.5$ mm. and had 55.6 per cent CH_3O .
 “ II “ “ 120–130°, $p = 0.5$ “ “ contained 49.75 per cent C and 7.83 per cent H.

Fraction II was heated at 80° in high vacuum for several hours and then distilled. This was repeated several times. A distillate was finally obtained, boiling between 115–120°. The syrup did not crystallize even on cooling. It reacted acid to litmus. 0.1081 gm. substance was titrated with 10 cc. of N NaOH and after standing at 50° for 2 hours, required 5.7 cc. of N acid for neu-

² Freudenberg, K., and Hixon, R. M., *Ber. chem. Ges.*, 1923, lvi, 2119.

³ Irvine, J. C., and Moody, A. M., *J. Chem. Soc.*, 1905, lxxxvii, 1462.

tralization. 0.1081 gm. of substance, therefore, neutralized 4.3 cc. of N NaOH equivalent to a molecular weight of 252. The substance analyzed as follows:

0.1052 gm. substance: 0.1938 gm. CO_2 and 0.0742 gm. H_2O .

0.1094 " " 0.4298 " Ag I.

$\text{C}_{10}\text{H}_{18}\text{O}_6$. Calculated. C 51.2, H 7.70, CH_2O 53.0.

Found. " 50.23, " 7.89, " 51.86.

It is evident that the material was the lactone containing a small proportion of the acid. However, on repeated fractional distillations, the composition remained constant.

This substance had the following rotations:

In Water.

$$\begin{array}{ll} \text{Initial.} & \text{Equilibrium.} \\ [\alpha]_D^{25} = \frac{+3.0^\circ \times 100}{1 \times 2.85} = +105^\circ & [\alpha]_D^{25} = \frac{+1.35^\circ \times 100}{1 \times 2.85} = +45.6^\circ \end{array}$$

The Optical Rotation of the Sodium Salt.

The substance was dissolved in 0.2 N NaOH.

$$[\alpha]_D^{25} = \frac{+1.04^\circ \times 100}{1 \times 2.512} = +41.6^\circ$$

The Optical Rotation of the Free Acid.

The substance was dissolved in 5 cc. of 0.4 N NaOH and allowed to stand at about 50° for 2 hours, cooled, and acidified with 5 cc. of 0.6 N HCl. The reading was taken immediately and the solution was then set aside at 50° . Final equilibrium was only reached after 24 hours.

$$\begin{array}{ll} \text{Initial.} & \text{After 2 hours.} \\ [\alpha]_D^{25} = \frac{+0.43^\circ \times 100}{1 \times 2.724} = +17.5^\circ & [\alpha]_D^{25} = \frac{+0.80^\circ \times 100}{1 \times 2.724} = +29.5^\circ \end{array}$$

$$\begin{array}{l} \text{After 24 hours.} \\ [\alpha]_D^{25} = \frac{+1.14^\circ \times 100}{1 \times 2.724} = +42.0^\circ \end{array}$$

STRUCTURE OF DIACETONE GLUCOSE.

SECOND PAPER.

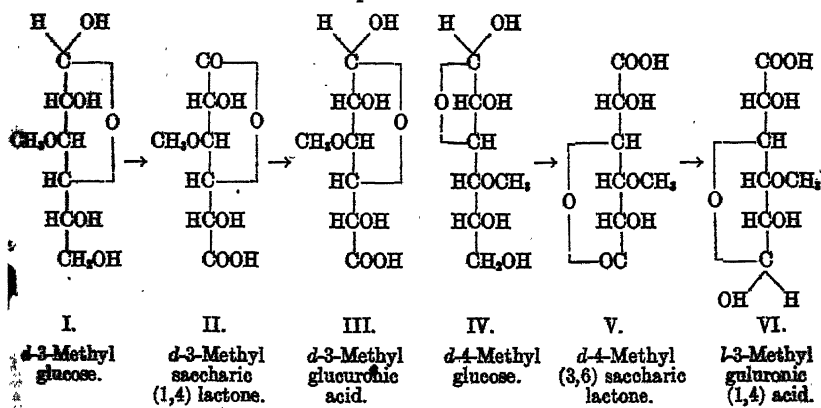
2-METHYL GLUCURONIC ACID AND 4-METHYL GLUCOHEPTONIC LACTONE.

By P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 17, 1924.)

Two important details in the structure of diacetone glucose are in dispute. One refers to the position of the second acetone group and the other to the nature of the oxidic ring. The first detail was satisfactorily solved by Levene and Meyer¹ as well as by Karrer² and seems now to be generally accepted. The second point is still under discussion. According to the view of so eminent an authority as Irvine,³ the oxidic ring has the structure of that of the γ sugars. On the other hand, Freudenberg and Doser⁴ attribute to it the usual butylene oxidic structure. H. Ohle⁵ in his very recent publication makes it clear that the evidence brought forth by either group of workers is not convincing. We also felt the need of more data before a final conclusion could be formulated. Hence the present work.



¹ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1922, liv, 805.

² Karrer, P., and Hurwitz, O., *Helvetica Chim. Acta*, 1921, iv, 728.

³ Irvine, J. C., and Patterson, J., *J. Chem. Soc.*, 1922, cxxi, 2146.

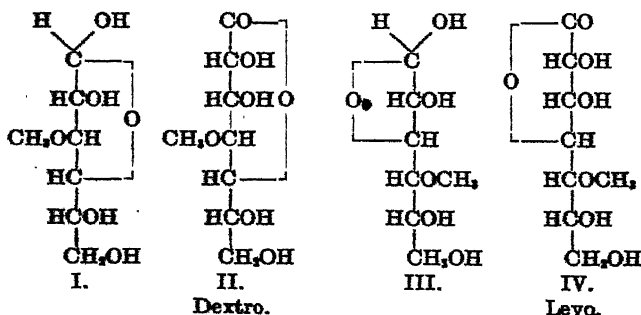
⁴ Freudenberg, K., and Doser, A., *Ber. chem. Ges.*, 1923, lvi, 1243.

⁵ Ohle, H., *Ber. chem. Ges.*, 1924, lxxv, 408.

The above graphic presentations indicate that the reduction of the monomethyl saccharolactone previously obtained by us on oxidation of monomethyl glucose could furnish evidence as to the position of the methyl group. If it is in position 3, then the resulting substance by I, II, and III should be *d*-3-methyl glucuronic acid. If it is in position 4, then by IV, V, and VI the resulting substance should be *l*-3-methyl guluronic acid.

The results of our present experiments point to the formation of *d*-3-methyl glucuronic acid. This conclusion is reached on the basis of the properties of the *p*-bromophenylhydrazine derivatives of glucuronic acid and of the new substance. The direction of mutarotation of the *p*-bromophenylhydrazine derivative of glucuronic acid is the same as that of *p*-bromophenyl glucosazone. It was pointed out by Levene and La Forge⁶ that the mutarotation of *d*-glucosazone shows a decline in the numerical value, the direction of the rotation being to the left. In this respect the *p*-bromophenylosazone of glucose and the corresponding derivative of glucuronic acid are identical. The mutarotation of each is in the sense of a decrease in numerical value and the direction is to the left. The new substance forms a *p*-bromophenylhydrazine derivative with practically the same properties as that of glucuronic acid and having the same character of mutarotation. If the substance had the structure of *l*-3-methyl guluronic acid, the mutarotation should have had the opposite character; namely, it should have shown an increase in the numerical value of its rotation as does the *p*-bromophenylosazone of gulose.⁷ For final proof, it is hoped eventually to prepare *l*-3-methyl guluronic acid.

A second source of information regarding the structure of the monomethyl glucose may be obtained from the heptonic acids derived from it.



⁶ Levene, P. A., and La Forge, F., *J. Biol. Chem.*, 1915, xxx, 429.

⁷ Levene, P. A., *J. Biol. Chem.*, 1915, xx, 429.

From these graphic formulas it is evident that 3-methyl glucose should yield a dextro-rotatory heptonic lactone, whereas 4-methyl glucose should lead to a levo-rotatory heptonic lactone. The methyl glucoheptonic lactone obtained from our methyl glucose is dextro-rotatory. The lactone obtained from unsubstituted glucose is levo-rotatory.

The synthesis of the 4-methyl heptonic lactone had still another theoretical interest. The sugars with oxidic rings other than the butylene oxidic acquire an increasing interest. Their synthesis depends upon the preparation of lactones having structures different from the 1,4-lactonic. Thus, it was important to inquire whether lactones form 4-methyl sugar acids. This question was answered in the affirmative through the present observation. The reduction of the lactone to the corresponding sugar is now in progress.

A second point of interest is the following. It was noted by Levene that in the sugar acids, the free acids and their salts rotate polarized light in opposite directions. The question arose as to whether this is a peculiarity only of those sugar acids that form 1,4-lactones, or whether the phenomenon is independent of the character of the lactone which the acid forms. It is seen from the present work that 4-methyl- α -glucoheptonic acid behaves in this respect as the non-substituted sugar acids. On the other hand, the tetramethyl mannonic acids show the same direction of rotation whether in the form of free acid or its salt.

EXPERIMENTAL.

Preparation of 3-Methyl Glucuronic Acid.—12.0 gm. of the crystalline methyl saccharolactone were reduced in the usual manner with a 2 per cent sodium amalgam. 25.0 gm. of the amalgam were required for the maximum reduction. Calculating on the basis of the reducing power of glucose, the yield of glucuronic acid was 20 per cent of the employed lactone. In view of the small quantity of material on hand, no attempt was made to obtain the crystalline methyl glucuronic acid. The reaction product was concentrated to a small volume, rendered slightly acid to Congo red by means of sulfuric acid, and the major part of the salt removed by the addition of 99 per cent alcohol. The filtrate was then concentrated to about 150 cc. An equal volume of water was added. One-half

of the solution, containing about 1.2 gm. of glucuronic acid, was then partially neutralized with sodium hydroxide until it reached acid to litmus, but not to Congo red. To this solution 3.5 gm. of *p*-bromophenylhydrazide hydrochloride and the same weight of sodium acetate were added and the solution was allowed to digest on a water bath. Soon the osazone began to crystallize in the form of a bright yellow flocculent mass, consisting of microscopic needles. After about 30 minutes, the first lot of the osazone was filtered and the filtrate was again placed on a water bath. The operation was repeated several times. All the precipitates were combined and suspended in ether to which a little acetic acid was added. The extraction with ether and glacial acetic acid was repeated until a sample, examined under the microscope, showed the absence of oil droplets. The yield of the dried material in this degree of purification was 0.700 gm. It was practically pure, as analysis has shown. However, it was once recrystallized from a mixture of alcoholic ether. The first product was the *p*-bromophenylosazone of *p*-bromophenylhydrazine glucuronate. In other words, the substance contained 3 equivalents of *p*-bromophenylhydrazine. The melting point of the substance was 157°C. and it analyzed as follows:

0.1006 gm. substance: 0.1516 gm. CO₂ and 0.0332 gm. H₂O.
 0.0971 " " 9.8 cc. nitrogen gas at *t* = 24°C. and *p* = 758.3.
 $C_{23}H_{17}O_8N_3Br_3$ Calculated. C 41.04, H 3.69, N 11.49.
 (Mol. wt. = 731.02). Found. " 41.09, " 3.69, " 11.56.

The optical rotation and the direction of mutarotation (in pyridine alcohol solution) are compared with the analogous derivative of glucuronic acid (prepared from saccharic acid lactone) and with that of glucose.

Derivative of 3-Methyl Glucuronic Acid.

Initial.	Equilibrium.
$[\alpha]_D^{25} = \frac{-0.52^\circ \times 100}{1 \times 0.5} = -104^\circ$	$[\alpha]_D^{25} = \frac{-0.07^\circ \times 100}{1 \times 0.5} = -14^\circ$

Derivative of Glucuronic Acid.

Initial.	Equilibrium.
$[\alpha]_D^{25} = \frac{-1.04^\circ \times 100}{1 \times 0.5} = -208^\circ$	$[\alpha]_D^{25} = \frac{-0.90^\circ \times 100}{1 \times 0.5} = -180^\circ$

Osazone of Glucose.

	Initial.		Equilibrium.
$[\alpha]_D^{20}$	$-\frac{0.40^\circ \times 100}{1 \times 0.5} = -80^\circ$		$[\alpha]_D^{20} = \frac{-0.03^\circ \times 100}{1 \times 0.5} = -6^\circ$

Osazone of Glucose.

	Initial.		Equilibrium.
$[\alpha]_D^{20}$	$-\frac{0.06^\circ \times 100}{1 \times 0.5} = -12^\circ$		$[\alpha]_D^{20} = \frac{-0.19^\circ \times 100}{1 \times 0.5} = -38^\circ$

3-Methyl Glucose.—It was found that 3-methyl glucose could be prepared by hydrolyzing 3-methyl diacetone glucose with sulfuric acid instead of hydrochloric acid. The use of silver carbonate was thereby avoided. 30 gm. of 3-methyl diacetone glucose were hydrolyzed with 600 cc. of 50 per cent alcohol, containing 1 per cent sulfuric acid in boiling water for 2 hours. An equal volume of boiling water is then added and the acid immediately neutralized with freshly precipitated barium carbonate. The solution is filtered with the aid of a little norit and concentrated to a syrup under diminished pressure. The syrup is taken up in hot methyl alcohol and filtered. 3-methyl glucose crystallizes from the filtrate on cooling. The yield including the quantities recovered from the mother liquor is about 50 per cent of the 3-methyl diacetone glucose used.

Preparation of d-4-Methyl- α -Glucoheptonic Lactone.

20.0 gm. of methyl glucose were dissolved in 40 cc. of water. 5 cc. of an 80 per cent solution of prussic acid and a few drops of ammonia were added. The solution was allowed to stand at room temperature for 4 days. It turned quite dark after 2 days. Another portion of 5 cc. of prussic acid was added and the solution allowed to stand 3 days. The further treatment was the usual in similar work. The final syrup on standing began to crystallize. The crystals were recrystallized from methyl alcohol and showed the composition of *d*-4-methyl glucoheptonic lactone. Its melting point was 204°C. and it had the following composition.

0.0996 gm. substance: 0.1570 gm. CO₂ and 0.0562 gm. H₂O.
 C₈H₁₄O₇. Calculated. C 43.22, H 6.35.
 Found. " 43.15, " 6.34.

The optical rotation in water was as follows:

Initial.	Equilibrium.
$[\alpha]_D^{25} = \frac{+0.96^\circ \times 100}{1 \times 2} = +48^\circ$	$[\alpha]_D^{25} = \frac{+0.07^\circ \times 100}{1 \times 2} = 3.5^\circ$

In 0.5 N NaOH and warmed to 50°C. for 2 hours.

$$[\alpha]_D^{25} = \frac{+0.14^\circ \times 100}{1 \times 0.1938} = +7.2^\circ$$

The substance was dissolved in 5 cc. of 0.4 N NaOH and kept at 50°C. for 2 hours. It was then cooled to 0°C. and acidified with 5 cc. of 0.6 N HCl. After the initial reading the solution was kept at 50°C. until equilibrium was established.

Initial.	Equilibrium.
$[\alpha]_D^{25} = \frac{-0.30^\circ \times 100}{1 \times 2.006} = -14.9^\circ$	$[\alpha]_D^{25} = \frac{+0.06^\circ \times 100}{1 \times 2.006} = +3.0^\circ$

Behavior of d-α-Glucoheptonic Lactone.

This substance was prepared according to Fischer. Its melting point was at 148°C. and it had the following composition:

0.1035 gm. substance: 0.1508 gm. CO₂ and 0.054 gm. H₂O.

C₇H₁₂O₇. Calculated. C 40.40, H 5.74.

Found. " 39.73, " 5.86.

The rotation of the substance in water was as follows:

Initial.	Equilibrium.
$[\alpha]_D^{25} = \frac{-1.12^\circ \times 100}{1 \times 2} = -56.0^\circ$	$[\alpha]_D^{25} = \frac{-1.00^\circ \times 100}{1 \times 2} = -50.0^\circ$

In 0.5 N NaOH:

$$[\alpha]_D^{25} = \frac{+0.08^\circ \times 100}{1 \times 2.012} = +3.98^\circ$$

The substance was dissolved in 5 cc. of 0.4 N NaOH and acidified with 5 cc. of 0.6 N HCl as previously described.

Initial.	Equilibrium.
$[\alpha]_D^{25} = \frac{-0.18^\circ \times 100}{1 \times 2.072} = -8.7^\circ$	$[\alpha]_D^{25} = \frac{-0.88^\circ \times 100}{1 \times 2.072} = -42.4^\circ$

ON THE SYNTHESIS OF HYDROXY AMINES BY THE CURTIUS METHOD.

BY P. A. LEVENE AND J. SCHEIDEGGER.

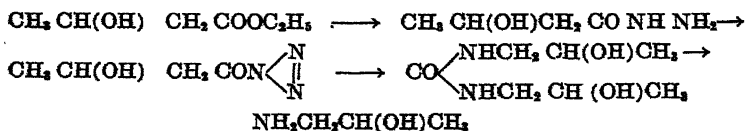
(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 17, 1924.)

In connection with the work on the structure of sphingosine it is desirable to prepare the higher hydroxy amines. A general method for preparing the lower members of this series consists in the condensation of aldehydes with nitromethane, with subsequent reduction. The higher aldehydes are not so easily prepared as the lower ones and neither are they so readily purified. On the other hand, higher fatty acids can be prepared in a high degree of purity. For this reason it seemed desirable to test whether β -hydroxy acids can be converted into 2-hydroxy amines by the method employed by Curtius for the preparation of amines. Should this synthesis be possible, then hydroxy amines with the hydroxyl in other positions will also be accessible by the same method.

The present communication contains a report on the preparation of 2-hydroxypropylamine by the Curtius process.

The starting point was acetoacetic acid which was reduced to β -hydroxybutyric, which was converted into the ester, and this into the hydrazide. The hydrazide was converted into 2-hydroxypropylurea, which was then hydrolyzed into 2-hydroxypropylamine.



EXPERIMENTAL PART.

Hydroxybutyric acid was prepared by the method of Wislicenus by the reduction of acetoacetic ester with sodium amalgam. It was converted into the ester in the usual way.

180 Hydroxy amines by the Curtius Method

β-Hydroxybutyric Hydrazide.—10.0 gm. were brought to a boil with reflux condenser. 13.0 gm. of ethyl-*β*-hydroxybutyrate were allowed to flow in very slowly from a dropping funnel. The solution was then kept boiling for about 30 minutes and then allowed to digest for 11 hours on a hot water bath. On cooling, the hydrazide crystallized in long needles. It was easily filtered and recrystallized from a little alcohol. It melted at 119–120°C.

0.1000 gm. substance: 20.6 cc. at $N_2 = 22^\circ C.$ and $p = 768.9$ mm.

$C_4H_{10}O_2N_2$. Calculated. N 23.7.

Found. " 24.12.

β-Hydroxybutyrylazine.—10.0 gm. of the hydrazide were dissolved in 8.7 cc. of concentrated solution of aqueous hydrogen chloride and transferred to a separatory funnel. To this solution were added 6.0 gm. of sodium nitrite in concentrated solution. A layer of ether was added to the solution of the hydrazide before the addition of the nitrite. The mixture was vigorously shaken. The extraction with ether was repeated several times. The ethereal extracts were then washed with water. The washed ethereal extract was taken up into 100 cc. of 50 per cent alcohol and allowed to remain on the water bath with reflux for about 18 hours. The solution was then concentrated under diminished pressure to a thick syrup. The substance crystallized on standing in the refrigerator. It was deliquescent. For purification, the crystals (filtered on suction at the temperature of about $+2^\circ C.$) were dissolved in ether. The ethereal solution was dried over sodium sulfate and dry ethereal solution allowed to evaporate in a vacuum desiccator over sulfuric acid. The residue crystallized when placed in a refrigerator. These crystals, however, were not analytically pure. For identification, it was convenient to convert them into the picrate.

For this the crystals were dissolved in an alcoholic solution of picric acid, containing a little more than two equivalents of the acid, and the solution was allowed to stand in a vacuum desiccator overnight. Heavy prisms settled out. The substance was recrystallized from water. It melted at 118–119°C. and had the following composition.

0.1000 gm. substance: 16.40 cc. N_2 at $t = 24^\circ C.$ and $p = 756.5$.

$C_{12}H_{12}N_4O_{11}$. Calculated. N 18.61.

(Mol. wt. 602.25) Found. " 18.75.

2-Hydroxypropylamine.—3.0 gm. of the crystalline urea derivative were taken up with 3.5 gm. of concentrated aqueous hydrogen chloride and heated in a sealed tube at 135°C. for 6 hours. The reaction product was concentrated *in vacuo* and an excess of the alcoholic solution of chloroplatinic acid was added. The platinic salt of the base soon crystallized. It was recrystallized from methyl alcohol. The substance contracted at 205° and melted at 210°C. It analyzed as follows:

0.1024 gm. substance: 0.0484 gm. CO₂ and 0.0346 gm. H₂O.

0.0964 " " 0.0357 " Pt.

C₆H₁₂O₂N₂PtCl₄·2HCl. Calculated. C 12.85, H 3.59, Pt 34.84.

Found. " 12.88, " 3.78, " 34.95.

THE ISOLATION OF ARACHIDONIC ACID FROM BRAIN TISSUE.

By LAURENCE G. WESSON.

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(Received for publication, March 18, 1924.)

Arachidonic acid, so named by Lewkowitsch,¹ is the tetra unsaturated, normal, aliphatic C₂₀ acid, which is present in brain tissue, in part at least, as one of the components of the phosphatides lecithin and cephalin. The present work was directed toward the easy isolation of this acid, free from other unsaturated acids, for use in further investigations.

The obstacles in the way of a successful isolation of arachidonic acid by any one of the few methods² by which this unstable acid has previously been obtained have been the difficulty of purifying the phosphatides, the difficulty of separating the various saturated and unsaturated acids of these phosphatides from one another, and finally the rapidity with which arachidonic acid becomes oxidized and resinified on exposure to air, light, and certain solvents. These difficulties are avoided in the following method by saturating the acid at an early stage with bromine, purifying the octobromarachidic acid thus obtained, and then debrominating the octobrom compound whenever it is desired to obtain arachidonic acid.

Method.

After dehydration of the ground tissue by means of acetone, it is thoroughly extracted in the cold with successive portions of

¹ Lewkowitsch, J., *Chemical technology and analysis of oils, fats and waxes*, London, 5th edition, 1913, i, 21.

² Hartley, P., *J. Physiol.*, 1909, xxxviii, 353. Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 359. Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1921, xlvi, 185; 1922, li, 285. Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlv, 193, 353; 1922, li, 507; 1922, liv, 91, 99.

U. S. P. ether. To the filtered ether extracts liquid bromine is added, a drop at a time, until a slight excess has been obtained. A white precipitate forms and slowly settles. After standing a few days, the supernatant liquid is removed, and the precipitate washed in the centrifuge with alcohol and ether until the washings are colorless. The precipitate is now digested at 37°C. for 24 hours with 50 per cent alcohol, containing 5 per cent hydrogen chloride, or at 50°C. for 1 hour with concentrated aqueous hydrochloric acid. After the digestion it is washed successively with water, alcohol, and ether, then dried at 100°C., and pulverized to pass a 200 mesh screen.

The product is a white, or nearly white powder, which has the composition of the octobromide of arachidonic acid. About 3.5 gm. are obtainable per kilo of wet brain tissue.

To obtain the tetra unsaturated acid from this product, copper-coated zinc dust is first prepared in the following way.³ In a flask provided with a ground glass-connected reflux condenser, a quantity of zinc dust (30 gm.) is treated with 1 per cent hydrochloric acid for a minute or two to remove the oxide from the surface. The zinc is allowed to settle, the dilute acid poured off, and successive portions of 0.5 per cent copper sulfate solution are added with shaking, each time allowing the zinc to settle and pouring off the supernatant liquid. When the zinc dust has become covered with a black coating of copper, it is washed by decantation in turn with 95 per cent alcohol and absolute alcohol. This copper-coated zinc dust may be used repeatedly.

1 gm. of octobromarachidic acid and 75 cc. of absolute alcohol are added to the copper-coated zinc. The alcohol is gently boiled while a very slow stream of dry hydrochloric acid gas is bubbled into the liquid. At the end of 1 hour the flask is cooled, and the alcoholic solution is decanted through a filter into a separatory funnel. 5 volumes of water are added, and the arachidonic acid (or ethyl ester) is extracted with ether. The ether is then washed with an equal volume of water and distilled from the arachidonic acid *in vacuo* without the application of heat.

In case free arachidonic acid rather than the ethyl ester is desired, the octobromarachidic acid is debrominated by copper-

³ Modified from Gladstone, J. H., and Tribe, A., *J. Chem. Soc.*, 1884, *xiv*, 154.

coated zinc dust and alcohol without the hydrochloric acid gas. 4 hours or more at the boiling temperature are required to attain approximately complete dehalogenation in this case.

DISCUSSION.

That the intermediate product described above as octobromarachidic acid is in reality this compound is indicated by the following facts: its elementary analysis agrees well with the calculated value (Experiment 1); its halogen content is substantially the same when prepared under widely varying conditions (Experiments 1 to 5); and when debrominated it yields arachidonic acid (Experiment 7).

The debrominated product (arachidonic acid or ester) is a colorless, or slightly yellow tinged liquid, possessing a tenacious, fishy odor. The free acid, when exposed as a thin film on glass to air and light for a day or so, forms a hard, resinous coating. It adds hydrogen to form arachidic acid (Experiment 7), and bromine to reform octobromarachidic acid (Experiment 6).

The question as to whether or not it is the same arachidonic acid (or acids), present originally in combined form in the tissues, cannot, of course, be answered. There may have been a shifting of the double bonds caused by the operations of adding and removing bromine, or a change in the spatial relationships in the molecule. However, we have little reason to suspect that the product obtained by this method is different from the arachidonic acid present as glyceride in the tissues, and the technical difficulties mentioned in a preceding paragraph, which have been encountered in other methods of isolating arachidonic acid from the tissues, are here to a large extent avoided.

EXPERIMENTAL PART.

Experiment 1. Bromination under Anhydrous Conditions.—Fresh hogs' brains were dried and extracted with ether as described above, and the ether was evaporated in *vacuo*. The residue in the flask was then dried by adding small portions of absolute alcohol and similarly evaporating them, after which it was dissolved in absolute ether. The ether solution was cooled, and bromine, which had been dried with concentrated sulfuric acid, was added, a drop at a time, until the color remained for 10 minutes. After 18 hours there was a solid, jelly-like mass in the flask, due to the compara-

tively small amount of ether used. Alcohol was added to facilitate centrifuging, and the procedure used from this point on was like that described under "Method" with aqueous hydrochloric acid digestion.

The thoroughly washed product was analyzed with the following results.

0.2 gm. substance: 0.1879 gm. CO_2 and 0.0595 gm. H_2O .

0.1 " " 0.1604 " AgBr.

$\text{C}_{10}\text{H}_{13}\text{Br}_2\text{O}_2$. Calculated. C 25.42, H 3.39, Br 67.75.

Found. " 25.62, " 3.33, " 68.26.

Experiment 2. Bromination of Partially Purified Cephalin.—Cephalin from sheep's brains was partially freed from lecithin by the method of Levene and Rolf⁴ until the ratio of amino nitrogen to total nitrogen was 75:100. This gives the cadmium chloride-cephalin compound, which was dried over sulfuric acid *in vacuo* in the dark for several days. To the chloroform solution of this product, a solution of bromine in chloroform was added in excess. After remaining in the ice box overnight, the chloroform was freed from bromine by an air current, and the chloroform distilled from the brominated compounds under reduced pressure.

Dilute alcoholic hydrochloric acid (2 per cent HCl) was then added, and the mixture allowed to stand at about 35°C. for 24 hours. The washed and dried product contained considerable cadmium and 65.1 per cent bromine. The calculated value for bromine in the cadmium salt of octobromarachidic acid ($\text{C}_{10}\text{H}_{11}\text{Br}_8\text{O}_2$) is 64.0 per cent. This product, which apparently is mainly the cadmium salt mixed with some free octobromarachidic acid, is then suspended in hot water, and the cadmium precipitated by means of hydrogen sulfide. The mixture of octobrom acid and cadmium sulfide was filtered off and digested with an excess of warm 10 per cent nitric acid which easily dissolved the cadmium sulfide. The residue was filtered off, washed, dried, and analyzed.

0.1 gm. substance: 0.1593 gm. AgBr.

$\text{C}_{10}\text{H}_{11}\text{Br}_8\text{O}_2$. Calculated. Br 67.75.

Found. " 67.79.

Experiment 3. Bromination of the Crude Phosphatide Mixture.—An ether extract of hogs' brain tissue was evaporated under reduced pressure to a small bulk, and alcohol and alcoholic cadmium chloride solution were added. The gummy precipitate was dried in a vacuum desiccator, dissolved in chloroform, and brominated. The precipitate was then separated, digested with alcoholic hydrochloric acid, washed, and dried as described above.

0.1 gm. substance: 0.1601 gm. AgBr.

$\text{C}_{20}\text{H}_{33}\text{Br}_2\text{O}_2$. Calculated. Br 67.75.

Found. " 68.14.

⁴ Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, liv, 93 (second method.)

Experiment 4. Bromination of the Same Product as in Experiment 3, but Digestion with Aqueous instead of Alcoholic Hydrochloric Acid.

0.1 gm. substance: 0.1594 gm. AgBr.

$C_{20}H_{32}Br_2O_2$. Calculated. Br 67.75.
Found. " 67.84.

Experiment 5. Bromination as Described under "Method."—The aqueous hydrochloric acid digestion was used.

0.1 gm. substance: 0.1599 gm. AgBr.

$C_{20}H_{32}Br_2O_2$. Calculated. Br 67.75.
Found. " 68.06.

Experiment 6. Rebromination of Arachidonic Acid.—0.5 gm. of octobromarachidic acid was debrominated as described under "Method," except that the zinc dust was not copper-coated. The ethyl ester of the unsaturated acid thus formed was extracted from the diluted alcohol in the manner described above, and treated in a cooled, absolute ether solution with a slight excess of bromine. The next day, when the solution had remained in the ice box overnight, the precipitate was separated, washed once with ether, and dried. The weight of the product obtained was 0.46 gm.

0.1 gm. substance: 0.1564 gm. AgBr.

$C_{20}H_{31}Br_2O_2C_2H_5$. Calculated. Br 65.84.
Found. " 66.56.

Experiment 7. Hydrogenation of Arachidonic Acid.—The method used was that of Skita and Meyer.⁵ 0.4 gm. of arachidonic acid, prepared from the octobrom addition product by copper-coated zinc dust without the use of hydrogen chloride, was reduced by hydrogen under a pressure of about 10 cm. of mercury, using colloidal platinum as the catalyst. The melting point of the crude product was 60°C. This was then crystallized once from acetone, and once from absolute ethyl alcohol. The melting point after the first crystallization was 65°C., and after the second 67°C. This partially purified acid was fused with an equal part of arachidic acid (melting point 68°C.), obtained from peanut oil by the method of Schweizer.⁶ The melting point of the mixture was 66°C., whereas admixture of an equal part of stearic acid, the melting point of which was 61°C., lowered the melting point to 57°C.

⁵ Skita, A., and Meyer, W. A., *Ber. chem. Ges.*, 1912, xlv, 3579.

⁶ Schweizer, A., *Arch. Pharm.*, 1884, xxii, 757.



A NOTE ON THE TECHNIQUE OF HEART PUNCTURE IN THE DOG.

By E. W. SCHULTZ.

(From the Department of Bacteriology and Experimental Pathology, Stanford University, California.)

(Received for publication, March 10, 1924.)

Although obtaining blood by heart puncture is a familiar procedure with smaller animals, it appears to be much less utilized in experiments on dogs than it would be if investigators appreciated its relative safety, convenience, and freedom from the hemorrhage and thrombosis that may result from repeated entrance into the vessels. Heart puncture is particularly convenient when repeated samples of arterial blood are taken.

If the bleeding is carried out as directed below, both the accidents and discomforts to the animal are negligible. We have been using this method over a year and during that time have taken more than 500 samples. The fatalities have been less than 1 per cent on the basis of the number of samples taken. These occurred in the early part of our work. The samples were taken from 43 dogs, some of which were bled as high as 45 times. If the entrance is properly made, little or no hemorrhage will take place into the pericardial sac or into the heart muscle. A heart bled repeatedly usually presents nothing more than small depressed puncture scars, with possibly a delicate deposit of fibrin. The individual punctures are usually confined to an area 2 cm. in diameter. The endocardium, except for the pitting, is usually smooth. No thrombi were found in any of the hearts. The actual traumatism is less than is usually experienced by bleeding from the veins, especially when daily samples are procured.

The animal is anesthetized or is held by an attendant, if anesthesia is contraindicated by the nature of the experiment. An area of about 4 inches in diameter overlying the apex of the

heart is shaved and painted with iodine. A carefully sharpened 18 gauge, 3 inch, Luer needle which has been boiled, is now attached to a syringe of suitable size. The needle is quickly forced through the skin and intercostal space. The point at which entrance to the thorax is made is important. The needle should enter a little below the apex of the heart, so that by making an angle of about 30° with the syringe and chest wall and pushing the needle forward, the apex of the left ventricle will be entered. This point is approximately where the axillary line crosses the fifth intercostal space. If the needle is gently pushed forward the impact with the heart is easily detected. Suction is then made as the needle continues forward. It should not be introduced further than is necessary to obtain the blood. This is done to avoid injury of the endocardium and heart muscle opposite the free point of the needle. Very little suction is necessary if the needle has been properly introduced. Indeed, the plunger may be forced up a short distance by the blood pressure. The object of entering the heart at the apex is to avoid hemorrhage from the larger coronary vessels and death from hemopericardium.

THE THERMODYNAMIC RELATIONS OF THE OXYGEN- AND BASE-COMBINING PROPERTIES OF BLOOD.

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In this paper the effect of temperature upon the oxygen dissociation curve of whole blood and its acid-base properties will be reported and an attempt will be made to explain these changes on a thermodynamical basis.

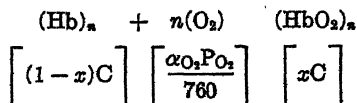
THEORETICAL.

The physicochemical status of whole blood coming within the purview of this paper includes the following facts or hypotheses which, on the basis of data already in the literature, are accepted with or without reservation.

1. The combination of oxygen with hemoglobin in whole blood is at least empirically predicted on the basis of Hill's (1910) well known formula. Slightly modified this is

$$K'O = \frac{[\text{HbO}_2]_n}{(1 - [\text{HbO}_2]_n) [\text{O}_2]^n} \quad (1)$$

The above equation is derived from the mass action law on the assumption that the mechanism of the oxidation of hemoglobin in whole blood is represented by the equation



At equilibrium $K'O$ is the oxygen dissociation constant of hemoglobin; $[\text{HbO}_2]_n$ is the concentration of the oxyhemoglobin; $1 - [\text{HbO}_2]_n$ is the concentration of reduced hemoglobin; and $[\text{O}_2]$

is the concentration of O_2 at equilibrium which at any one temperature may be expressed as partial pressure. " n " is a positive whole number or fraction which for pure hemoglobin in the absence of salts is 1; and in the presence of salts varies from 1.5 to 3.5. For whole blood it is 2.5 ± 0.3 .

By mass action we employ concentrations as indicated above.

$$K'O = \frac{[xC]}{\left[(1-x)C\right] \left[\frac{\alpha_{O_2} P_{O_2}}{760}\right]^n} = \frac{x}{(1-x) \left[\frac{\alpha_{O_2} P_{O_2}}{760}\right]^n} \quad (2)$$

Where C is the concentration of hemoglobin, x is the fraction of saturation with oxygen, α_{O_2} is the solubility of O_2 in the system at $t^\circ C.$, and P_{O_2} is the partial pressure of oxygen.

As ordinarily calculated in terms of partial pressure instead of concentration of oxygen the oxygen dissociation constant has a different value.

$$K_O = \frac{x}{(1-x)P_{O_2}^n} \quad (3)$$

This is the usual form of Hill's equation.

Since the mass action law requires the active masses to be expressed in terms of concentration the correct equilibrium constant should be so calculated. So long, however, as we are working at one temperature it is of little significance which method of calculation is used since only the order of magnitude of the equilibrium constant will be changed by either choice.

However, it is obvious that at different temperatures the partial pressures are not comparable and hence the equilibrium constants must be expressed in concentration terms since it is the active mass in solution with which the hemoglobin is in equilibrium. It is perhaps more convenient to retain K_O in terms of partial pressure since when necessary it may be easily converted to $K'O$, the equilibrium constant in concentration terms. It is obvious from equations (2) and (3) that

$$K'O = \frac{K_O}{\left(\frac{\alpha_{O_2} P_{O_2}}{760}\right)^n}$$

or

$$\log \frac{1}{K'O} = \log \frac{1}{K_O} + n \log \left(\frac{\alpha_{O_2} P_{O_2}}{760} \right) \quad (4)$$

The values of this conversion factor at 15 and $38^\circ C.$ are given in Table I. The difference in constants as calculated by the two methods corre-

sponds to a heat of reaction of 6,700 cal. or 2,800 cal. per mol. of oxygen which is obviously the molecular heat of solution of oxygen.

This follows because for K_O the equilibrium occurs between gaseous oxygen and hemoglobin in solution and thus the heat effect of the solution of O_2 is included. For $K'O$ the equilibrium is between oxygen in solution and hemoglobin in solution so that the heat of solution of oxygen is not included. The calculation is made by substituting the value of the conversion factors into the van't Hoff isochore.

No rigid explanation of the significance of " n " has been given. It has been called by Barcroft (1914) and Brown and Hill (1923) the "degree of aggregation" of hemoglobin. The addition of

TABLE I.

Factor to Convert $\log \frac{1}{K_O}$ in Partial Pressure Terms to Concentration Terms
for Whole Blood.

$$n = 2.4$$

t	$650\alpha_{O_2}^*$	$\log \frac{650\alpha_{O_2}}{760}$	Corresponding difference in heat reaction cals.
15	31.22	-3.325	6,700
38	21.84	-3.700	

* Van Slyke's (1922) value of 0.65 for the relative solubility of gases in red blood cells $1,000 \times 0.65 \alpha_{O_2} = \text{cc. of } O_2 \text{ dissolved per liter of red blood cells.}$

salts to pure hemoglobin requires the introduction of values of " n " greater than 1 into Hill's formula in order that the calculated oxygen dissociation curve may fit the observed. In other words, there is an apparent change in the order of reaction. It is difficult to see why the aggregation of one reactant should necessarily change the order of reaction. In reactions involving substances such as water, benzoic acid, etc., which vary markedly in degree of aggregation under varying conditions no change in the order of their reactions is observed under these varying conditions.

2. Further, it is assumed that (1) " n " is constant for any one blood over a wide range of pH, and (2) is independent of the oxygen tension (degree of saturation). These two assumptions are here tentatively accepted.

For the first it is difficult to see why \bar{H} should be free of the aggregating power exerted by metallic ions. For the second there is a strong suspicion hinted by Henderson (1920) that " n " varies with the degree of saturation (and therefore oxygen concentration) at constant pH. At any rate there is reason to believe that the explanation implied by " n " in Hill's equation does not clear up the chemical mechanism of the combination of oxygen and hemoglobin in the presence of salts. In this connection it might be mentioned that Wilson (1923) found the osmotic pressure of pure hemoglobin increased fourfold with the \bar{H} concentration and, further, Camis (1922) reports changes in degree of aggregation produced by \bar{H} . More significant are the findings of Hartridge and Roughton (1923) who studied the velocity of reduction of oxyhemoglobin. They found that for both dialyzed hemoglobin and in the presence of salts the reaction is a monomolecular one. Apparently, then, from this direct observation salts do not change the order of reaction of oxygen with hemoglobin.

On the other hand, Brown and Hill (1923) have recently attempted to substantiate equation (2) on thermodynamical grounds as representing the true mechanism of the oxidation of hemoglobin.

Brown and Hill¹ have sought to determine " n " independently of all assumptions. "If blood be exposed to a gas mixture containing a given concentration of oxygen at a temperature T_1 , and a sample taken having a degree of saturation y_1 , and then the same (or similar blood) be exposed to the same gas mixture at constant volume at another temperature T_2 , and another sample taken given a degree of saturation y_2 , we may write

$$\frac{y_1}{(1-y_1)} - K_1 x^n = \frac{y_2}{(1-y_2)} - K_2 x^n$$

From these we may eliminate x and n completely by division, so obtaining

$$\frac{K_1}{K_2} = \frac{y_1(1-y_2)}{y_2(1-y_1)}$$

From $\frac{K_1}{K_2}$ we may now calculate Q_O the heat of absorption of oxygen per one mol (Hb)₂ of hemoglobin with n mols of O_2 . From independent direct measurements of the heat of oxidation per mol of oxygen q is obtained. It is evident that $n = \frac{Q_O}{q}$. Hill found

$$n = \frac{Q_O}{q} = 2.80 \pm 0.13$$

It must be pointed out, however, that " n " is here assumed to be constant for one oxygen pressure and no more. The conclusion then has no more validity than this: that at constant oxygen concentration the active masses of hemoglobin and oxyhemoglobin are different from the apparent active masses in the ratio

¹ Brown and Hill (1923), p. 312.

$$\frac{(\text{HbO}_2)}{1 - (\text{HbO}_2)} : \sqrt[2.8]{\frac{(\text{HbO}_2)}{1 - (\text{HbO}_2)}}$$

Two difficulties still remain in Hill's theory of the combination of oxygen by hemoglobin in whole blood: (1) the change of the order of reaction by the introduction of salts, and (2) the independence of the affinity of oxygen at constant C_H of the amount of oxygen combined.

3. Increase of acidity of whole blood causes a diminution in the value of K_O ; *i.e.*, the affinity of oxygen for hemoglobin is less. Quantitatively the effect has been expressed by the relation

$$\left(\frac{\delta \log \frac{1}{K_O}}{\delta \text{pH}} \right) = \text{constant}$$

This equation rests at present solely upon the experimental results of Barcroft (1914), Donegan and Parsons (1919), and others.

They found that the values of $\log \frac{1}{K_O}$ plotted against pH over narrow ranges of pH (approximately 7.0 to 8.0) gave straight lines. These were called by Hasselbalch, "Peters-Barcroft" lines or more simply "P-B" lines. The thermodynamic derivation of the relation between K_O and pH will be given later.

4. The effect of temperature upon K_O is inverse; *i.e.*, at a lower temperature the affinity of oxygen for hemoglobin is greater. Quantitatively, the validity of van't Hoff's isochore for whole blood is accepted and the relation

$$\left(\frac{\delta \ln K_O}{\delta T} \right)_{\text{pH}} = \frac{Q_O}{RT^2}$$

which in the integrated form, employing 10 base logs, is

$$\log \frac{K_O}{K'_O} = \frac{Q_O(T - T')}{4.6 TT'}$$

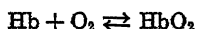
where Q_O is the heat of absorption of oxygen by blood per mol of "aggregated" hemoglobin, gives the variation of K_O with temperature *at constant pH*.

The validity of this assumption rests upon the agreement between Q_O thermodynamically calculated from the equilibrium constants and directly determined by Barcroft and Hill (1910) and Brown and Hill (1923).

Note carefully that Q_0 as above defined is the heat of *absorption* of oxygen by blood (or hemoglobin solution) per 1 mol of "aggregated" hemoglobin and should be distinguished from the molecular heat of *oxidation* of hemoglobin *per se* which is an entirely different quantity.

The former included the latter and many other heat effects besides. Further, it is well to indicate here that 1 mol of hemoglobin (Hb)_n in Hill's sense is "*n*" mols in the ordinary terminology. Wherever any confusion might arise in this paper the distinction will be pointed out by using the term "aggregated."

5. The hemoglobin molecule combines chemically with a maximum of 1 molecule of oxygen. Disregarding for the moment the question of aggregation or polymerization the reaction



represents a true chemical reversible reaction as distinguished from "adsorption."

The basis for this assumption rests mainly upon (1) Peters' (1912) observations that the relation of iron to oxygen in oxyhemoglobin is always exactly 1 atom to 2 and (2) the well known specificity of the absorption spectra for reduced oxy- and carbon monoxide hemoglobin.² (3) More significant is the fact that the maximum combination of oxygen with hemoglobin by weight is 0.0019 gm. of O_2 per gram of hemoglobin or 32 gm. of O_2 per 17,000 gm. of hemoglobin. Similarly, carbon monoxide combines in the ratio of 28 gm. of CO per 17,000 gm. of hemoglobin. These ratios hold for many different species of hemoglobin. Osmotic pressure determination of pure hemoglobin gives a molecular weight of approximately 17,000 so that the combination of O_2 and CO with hemoglobin is a 1:1 molecular ratio. Now an adsorption process may have an asymptotic limit but only mere chance could fix this limit at a 1:1 molecular ratio. The probability that two dissimilar gases could by chance have in an adsorption process the same molecular limiting ratio is too remote for consideration.

6. In addition to binding oxygen hemoglobin binds base and functions over physiological ranges of pH as a polybasic acid, containing an indeterminate number of acid groups. Van Slyke

² See discussion in *Nature* (*Nature*, 1923, xxi, 496 and following).

(1922) has shown that the *minimum* number of acid groups in the polybasic acid hemoglobin active over 1 unit of pH is about 5. Consideration of Fig. 1 shows that the actual number is probably more than the minimum and as outside limits gives 7 to 15. Over a pH range of 6.5 to 7.5 at 38°C. the pk' values of these groups vary from 7.7 to 8.9. In the limiting case these pk' values would be about equally spaced so that (approximately)

$$pk'_1 - pk'_2 = pk'_2 - pk'_3 = \dots < 0.1$$

7. The total base at any pH bound by reduced hemoglobin is the sum of the base bound to each radical; *i.e.*,

$$B_R \text{ (total)} = B_{1R} + B_{2R} + B_{3R} \dots$$

8. There is strong presumptive evidence that hemoglobin binds oxygen only at one portion of the molecular; *viz.*, the heme portion. The reaction is intimately concerned with an iron atom and a carboxyl (or other acid) group. By virtue of the introduction of electronegative oxygen in close proximity to one acid radical the ionization of this "oxylabile" radical as an acid is increased. The degree of increase is, as calculated from the data of Haldane, Doisy, Van Slyke, and others,

$$\frac{k_o}{k_r} = 20 \pm 10$$

where k_o and k_r is the ionization constant of the acid radical in question in the oxidized and reduced form, respectively. As before, the base bound by oxyhemoglobin is the summation of the base bound to each radical or

$$B_O \text{ (total)} = B_{1(O)} - B_{1(R)} + B_{2R} + B_{3R} + \dots$$

In other words, oxyhemoglobin combines at constant pH with $(B_{1(O)} - B_{1(R)})$ more base than reduced hemoglobin.

The assumption that one and only one of the acid radicals of reduced hemoglobin has its ionization constant changed upon oxidation has been tacitly made by Henderson (1920) and Hill (1923) without rigorous proof. As a corollary to Section 5 above it is a natural and conceivable hypothesis that if but 1 molecule of oxygen combines with 1 molecule of hemoglobin its sphere of in-

fluence would be limited. In the hematin portion of the molecule this influence might be so limited as to affect one acid group alone. That the introduction of oxygen close to a carboxyl does increase the ionization of the acid group is well known; *e.g.*, the change of propionic to lactic acid (as cited by Parsons) gives

$$k_{\text{lactic}} \quad 10$$

The proof of this assumption can be furnished in two ways. (1) By calculating $B_{1(O)} - B_{1(R)}$ and checking the calculated values with the observed. (2) By calculating the values of K_O on the basis of the assumption and comparing with observed K_O . Both types of proof are presented in this paper.

Van Slyke³ has unpublished data at 38°C., in addition to his published data of 1923, on the value of $B_O - B_R$ over a wide range of pH in proof of this assumption.

9. The total titration curve of reduced whole blood, BHb plotted against pH or BHCO_3 against pH, is a straight line; *i.e.*,

$$\left(\frac{\delta \text{BHb}}{\delta \text{pH}} \right)_t \cdot \text{constant} = \beta_R C$$

Where β is the molecular buffer of reduced hemoglobin and C = concentration. β_R is 3.0 ± 0.5 mols of base.

The basis for Sections 6, 7, 8, and 9 rests upon the early work of Hasselbalch and Lundsgaard (1912); Christiansen, Douglas, and Haldane (1914); Parsons (1920); Henderson (1920); and more recently that of Van Slyke (1922). Further corroboration is given in this paper.

10. For oxyhemoglobin the total titration curve is concave to the pH coordinate and the expression

$$\left(\frac{\delta \text{BHbO}_2}{\delta \text{pH}} \right)_t = \beta_R C + \left(\frac{\delta (B_{1(O)} - B_{1(R)})}{\delta \text{pH}} \right)_t C \quad (6)$$

represents the relation between base bound and pH.

Fig. 1 is a schematic illustration of the statements made under Sections 7 to 10. The ordinates are mols of base bound by hemo-

³ Personal communication.

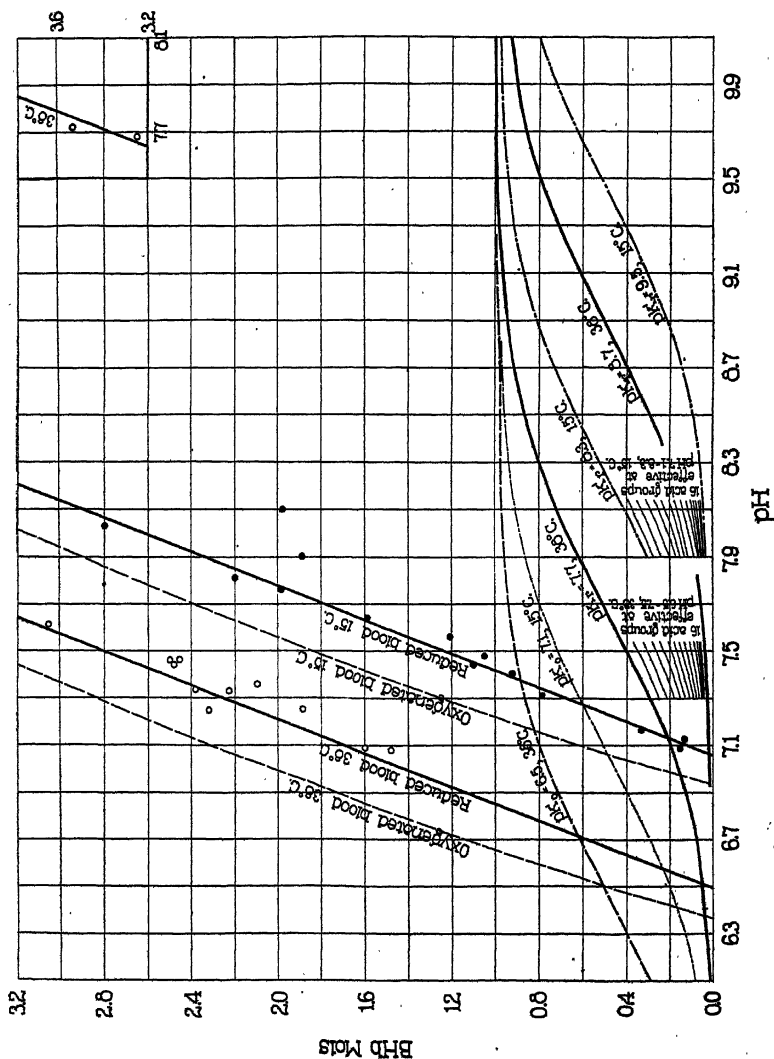


Fig. 1. The titration curves of polybasic hemoglobin-acid in whole blood at 38 and 15°C. The heavy curved lines are the titration curves of the strongest and the weakest acid groups effective at 38°C. between pH 6.5 to 7.5. Between these two about sixteen acid groups of intermediate strength function. The total base bound by these sixteen acid groups at any given pH gives the titration curve of reduced hemoglobin at 38°C. Oxygenation increases the strength of one acid group so that its titration curve is moved toward the left. The total base bound by this group plus the remaining unaffected groups give the titration curve of oxyhemoglobin which is concave toward the abscissa. Decrease in temperature decreases the ionization of all the acid groups of hemoglobin-acid. This results in a displacement of all the curves to the alkaline side. A similar diagram is shown for the acid groups and whole blood at 15°C. experimental points are shown as dots and circles.

globin; the abscissæ pH. At pH 6.5 (approximately the isoelectric point at 38°C.) no base is bound. If whole reduced blood be titrated at 38°C. the titration curve will be a straight line the slope of which is $\beta_x = 2.8$ or the mols of base taken up by a mol of hemoglobin over a pH change of 1 unit. Since hemoglobin is a polybasic acid we may draw the titration curves of the individual acid groups. The first of these is represented by a heavy line. Now it is evident from the titration curve that a weak acid combines with practically no base at a pH 1.2 units on the acid side of its pk' . It follows that the strongest acid in hemoglobin active at pH 6.5 has a pk' of $6.5 + 1.2 = 7.7$. Similarly, at pH 7.5 no acid group weaker than $pk' = 8.7$ can bind appreciable base. In other words, the acid groups effective between pH 6.5 to 7.5 have pk' values between 7.7 and 8.7. Since from the curve the strongest binds only 0.4 mol of base at pH 7.5 and the others bind less it may be readily seen that for one limit, if about sixteen acid groups whose pk' values are equally spaced are assumed to function between these limits, approximately 2.8 mols of base will be bound by them at pH 7.5. This, of course, is β_x the amount bound by whole reduced blood over 1 pH unit. At all points between 6.5 to 7.5 the locus of the sums of the bases bound by the sixteen acid groups will form a straight line which is the total titration curve of whole blood. That the pk' values of these acid groups are approximately equally spaced follows because of the straightness of the whole blood titration curve. As the contrasting limit we may conceive of seven acid groups whose pk' values are the same, *i.e.* 7.7, in which case the total titration curve would be an elongated single titration curve with a double curvature, which would sufficiently deviate from straightness to be experimentally detected.

This description is by no means intended to represent the exact condition of the acid groups in hemoglobin but rather to give the approximate conceivable limits. Thus it may be said that there are from seven to sixteen acid groups in hemoglobin active over 1 pH unit. The pk' values of these groups vary from identity to each other to values increasing by approximately equal increments which approach 0.1 in value. Since the titration curve of reduced hemoglobin is a straight line over a considerable pH range the later condition is probably nearer the real one.

When oxygen combines with hemoglobin *one* acid group increases its ionization. In consequence the titration curve of this "oxy-labile" group is shifted 1.2 units toward the acid side since $\log \frac{k_o}{k_r} = 1.2$ (approximately). Oxyhemoglobin then combines with more base at any given pH by an amount

$$\Delta B_0 - R = B_{1(o)} - B_{1(r)}$$

The titration curve of oxidized whole blood is then drawn by adding to the curve for reduced blood these increments of base. As shown it becomes a curve concave to the abscissa.

11. The dissociation constants of the acid radical of hemoglobin change with temperature proportionately to the heat of ionization. The validity of the van't Hoff isochore is assumed so that

$$\frac{d \ln k_{acid}}{dT} = \frac{q_{acid}}{RT^2} \quad (7)$$

represents the change of any k_{acid} of hemoglobin with temperature.

q_{acid} is the heat of ionization per equivalent of H^+ . The change of dissociation constant for all the acid radicals with temperature is approximately the same.

Proof of these assumptions is offered here for the first time.

12. The dissociation constant of carbonic acid changes in blood with temperature according to the same relation.

13. In blood the two acids chiefly concerned in the acid-base equilibrium are carbonic and hemoglobin-acid. All base shift with varying pH at any temperature may be assumed for most of the purposes of this paper to involve changes in these two acids.

14. The base chiefly concerned in the acid-base shift is sodium. For the purpose of this paper all others are neglected.

15. The isoelectric point of reduced hemoglobin is about pH 6.8 at 20°C. At this point by definition it combines with minimum base. For hemoglobin this is practically zero.

EXPERIMENTAL.

The blood used was the whole blood of W. C. S. and was drawn usually in the morning, using as little stasis as possible. It was oxalated with neutral potassium oxalate to 0.2 per cent and kept in

For saturation in tonometers under known conditions the method known as the "first saturation method" of Austin et al (1922) was used. One or more saturations were done at $15 \pm 0.1^\circ$ or $38 \pm 0.1^\circ$ according to the nature of the experiment. The analyses of the gas phase were done using the Haldane-Henderson gas apparatus.

The blood was transferred to mercury receivers and analyzed, with precautions to prevent loss or gain of gases.

The blood gases (CO_2 and O_2) were determined in duplicate on the Van Slyke constant volume blood gas apparatus.

The pH values of the plasma at 15°C . were determined, using Cullen's (1922) colorimetric method. The readings were done with both diluted plasma and standards at 15°C . Inasmuch as the empirical correction for the colorimetric method has not been determined at 15°C . the results are reported as read + 0.04, which correction was obtained from Cullen's data by extrapolation. The pH values at 38°C . are calculated, using a constant pK_1 of 6.15.

DISCUSSION.

Part I. Thermodynamic Relations of the Base-Binding Properties of the Blood.

1. *Determination of the Change of $pK_{\text{H}_2\text{CO}_3}$ for Whole Blood with Temperature, $\frac{dpK_{\text{H}_2\text{CO}_3}}{dT}$.*—The value of the dissociation constant of carbonic acid is known to change with temperature in aqueous solutions. This change has been measured accurately by conductivity measurements and from these measurements the heat of ionization of H_2CO_3 has been calculated from the relation

$$\frac{d \ln K_{\text{H}_2\text{CO}_3}}{dT} = \frac{q_{\text{H}_2\text{CO}_3}}{RT^2}$$

to be $-2,800$ cal. (Landolt-Börnstein). The order of this change in whole blood was determined in Experiment 1, from determinations of the value of pK_1 at 15°C . The average value obtained was 6.27 ± 0.05 .

This value of pK_1 practically agrees with that of Warburg (1922) determined at 18°C . who used a hydrogen electrode. Together with Warburg's and Hasselbalch's (1911) results it is apparent that

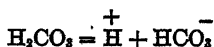
the pK_1 of carbonic acid in whole blood is greater at 15°C. than at 38°C. and of the order of magnitude which would be calculated from the heat of ionization using $pK_1 = 6.15$ at 38°C. which latter value is well established by Cullen (1922) and others.

The assumption then that the van't Hoff isochore gives the variation of $k'_{H_2CO_3}$ with temperature in whole blood is justified. In all subsequent calculations a pK_1 of 6.30 for whole blood at 15°C. is used. These values are calculated from the values at 38°C. $pK_1 = 6.15$ for whole blood using $-2,800$ cal. as the heat of ionization of carbonic acid.

The use of a constant pK_1 for whole blood at 15 and 38° over a range of pH of about 1 unit and at varying degrees of saturation is warranted only because the results reported here are comparative. Factors changing pK_1 at one temperature, would likewise change it in the same direction at the other temperature. There is no reason to believe that variations in the magnitude of these changes at different temperature and at any pH would alter the general conclusions.

The authors realize that the use of an extrapolated correction of $+0.04$ pH from Cullen's data on the colorimetric pH at 20°C. to 15°C. is uncertain. It has been used in lieu of other data. Any future determinations at 15°C., if anything, would tend to diminish this correction so that the pK_1 value at 15°C. found here would be less. This would result in a calculated heat of ionization of carbonic acid less than $-2,800$ cal. There is no *a priori* reason for believing that the heat of ionization in blood would be different from that in aqueous solutions.

In fact the heat calculated from the equilibrium constant represents the change of free energy of the reaction



when the reaction goes completely from left to right. This must be the same regardless of where or how the reaction is brought about. If the heat so calculated for blood from the experimental data does not agree with the heat as measured in aqueous solutions, the inevitable conclusion reached is that the dissociation of carbonic acid in blood is different from that in water. The weight of evidence is against this last hypothesis. Therefore, the agreement of the value found here with that of Warburg and Hasselbalch and the agreement of the heat of ionization of H_2CO_3 calcu-

lated for blood from these data with that found by Walker and Cormack (1900) for aqueous solutions of carbonic acid make these values close approximations to the real ones.

A criticism offered by Professors J. H. Austin and G. E. Cullen, of the Department of Research Medicine, University of Pennsylvania, who reviewed this paper in the manuscript form must be discussed here. While they do not object to the validity of the van't Hoff isochore for carbonic acid in blood they rightly maintain that the agreement of the heats of ionization in blood and in water obtained by E. M. F. measurements is, quite aside from matters of technique, not a rigid proof. This arises because the hydrogen ion concentration determined by the hydrogen electrode at any temperature is not the true hydrogen ion concentration, but rather the square root of the activation product of the reference substance. To make this clear, if the reference $\overset{+}{\text{H}}$ standard be 0.1 molar HCl, E. M. F. measurements at any given temperature give the activity of HCl; *i.e.*,

$$a_{\text{HCl}} = \sqrt{a_{\text{H}} \cdot a_{\text{Cl}}}$$

We can only make this equal to $\overset{+}{\text{H}}$ by assuming $a_{\text{H}}^{\pm} = a_{\text{Cl}}$. This, of course, is unwarranted since the activities of $\overset{+}{\text{H}}$, the hydrogen ion, and chlorine ion are known to be unequal. So long as we are working at one temperature this is of little consequence, but in changing to another temperature a further difficulty arises since, although we know the change of activity of HCl with temperature, we do not know the change of activity of the individual ions. There is reason to believe that the rate of temperature change is different for the two ions. At the second temperature as before we are compelled to make the same assumption and we compare at the two temperatures, not hydrogen ion concentrations but square roots of activation products. We cannot escape this dilemma until the activity of the individual ions is measurable. The rigorous proof of the validity of the van't Hoff isochore for H_2CO_3 in blood (as well as in water) must wait upon this. We can accept it for the present on pure thermodynamic grounds with the assurance that its use will necessitate no future violent changes in the general conclusions reached.

If the pK_1 for carbonic acid at 15°C . used here be subsequently found to be too high the heat of ionization of hemoglobin calculated in the following section would be too high. However, these considerations would in no wise affect the method of treatment of the subject employed here nor the general conclusions reached.

2. *Calculation of the Heat of Ionization of Hemoglobin-Acid per Equivalent of \bar{H} .*—In Fig. 1 are drawn the titration curves of all the acid groups of the weak polybasic acid-reduced hemoglobin (of which there are at least sixteen effective at pH 6.5 to 7.5) at 38°C . The total titration curve of these acids will be the sum of the individual curves and will be the titration curve of blood. This is represented by a line drawn through the experimental points. For any one acid group at any pH we may put for its fraction of the total base bound

$$B_1Hb = \frac{k'_{aa}}{k' + H} C^* \quad (8)$$

If the temperature be lowered to 15°C . the titration curves of all the acid groups will be shifted to the acid or alkaline side according as the acid becomes weaker or stronger; *i.e.*, according as the heat of ionization is positive or negative. If the heat of ionization be the same for all groups the shift of each will be the same. This in the light of Meyerhof's (1922) results on the heat of ionization of various protein acids is a probable assumption. The total titration curve of the blood will be likewise shifted. In Fig. 1 it is

$$\frac{k'}{H} = \frac{BA}{HA}$$

If C is the total concentration of free and combined acid

$$HA = C - BA$$

so that

$$\frac{k'}{H} = \frac{BA}{C - BA}$$

or

$$BA = \frac{k'}{k' + H} C$$

* This equation is derived from Henderson's equation.

represented by a line drawn through the experimental points. k'_{ac} has changed to $\varphi k'_{ac}$, where by van't Hoff's isochore in exponential form

$$\varphi = \frac{k'_t}{k'_{t'}} = e^{\frac{q_{ac}(T_1 - T_2)}{RT_1T_2}} \quad (8a)$$

q_{ac} being the heat of ionization of the acid group. As before for the one particular acid group in question the base bound is

$$B_1HB = \frac{\varphi k'_{ac}}{\varphi k'_{ac} + H_{t'}} \quad (9)$$

Equating (8) and (9) and solving for $\log \varphi$ we have

$$\log \varphi = \text{pH}_t - \text{pH}_{t'} \quad (10)$$

from which it follows that for a polybasic acid the horizontal shift of the total titration curve to the acid or alkaline side for a given change of temperature is proportional to the change of the ionization constant and hence the heat of ionization. Equations (8 a) and (10) allow us to calculate q_{ac} , the heat of ionization of hemoglobin-acid per equivalent of hydrogen, from Fig. 1 which gives the shift of the total titration curve of reduced hemoglobin in whole blood and hence the shift of each acid radical. The data and calculated heat of ionization are shown in Table II. This value is in complete accord with the directly determined heats of ionization of protein acids (—11,000 to —13,000 cal.) determined by Meyerhof. As a corollary the assumption that the heats of ionization of all the acid radicals in hemoglobin active over pH 6.5 to 8 (approximately) are identical is verified. For if one or more acid radicals should change disproportionately to the others, the titration curve of whole blood at 15°C. could not be of the same form as at 38°C.; i.e., a straight line.

The points plotted in Fig. 1 are obtained from Experiments 1 and 2 and were calculated as follows:

The mols of base bound to the hemoglobin were calculated by assuming that total available base of whole blood to be equivalent to 95.0 volumes per cent of CO_2 . From this value, the amount of NaHCO_3 found and the oxygen capacity, the mols of base bound to the hemoglobin are readily calculated.

$$\frac{95 - \text{NaHCO}_3}{\text{oxygen capacity}} = \text{Mols of base bound to 1 mol of hemoglobin.}$$

Since the bloods were at varying degrees of saturation with oxygen the values were corrected to complete reduction, using the ΔB_{O-R} (Fig. 2) values determined separately in Experiment 3. The base bound by reduced blood is given in the last column of the table for Experiments 1 and 2.

The propriety of assuming a constant amount of available base is warranted by two considerations: (1) the known relative constancy of the available base in the blood of normal individuals, and (2) the fact that the same conclusions are reached if the experimental points are plotted as $BHCO_3 - pH$ without assumption as to base. The exposition of concentration in molar terms rather than volumes per cent is clearer and more general.

3. *Effect of Temperature on the Buffer Value of the Whole Blood. Change of pH of Maximum Buffer with Temperature.*—The molecular buffer β_m of any acid is

$$\beta_m = 2.3 \frac{k'_a \overset{+}{H}}{(\overset{+}{H} + k'_a)^2}$$

As Van Slyke (1922) has shown this has a maximum value when its first differential

$$\left(\frac{\delta \beta_m}{\delta pH} \right)_{t^0} = (2.3)^2 \overset{+}{H}_t \frac{\left(\overset{+}{H}_t - k'_a \right)}{\left(k'_a + \overset{+}{H}_t \right)^3}$$

is zero the condition for which is fulfilled when $\overset{+}{H} = k'_a$. Similarly, at any other temperature t'

$$\left(\frac{\delta \beta_m}{\delta pH} \right)_{t'} = (2.3)^2 \overset{+}{H}_{t'} \frac{\left(\overset{+}{H}_{t'} - k'_a \right)}{\left(k'_a + \overset{+}{H}_{t'} \right)^3}$$

β_m is maximum when $\overset{+}{H}_{t'} = \varphi k'_a$

As before $\varphi = \frac{k'_{t'}}{k'_a} = e^{\frac{q(T'-T)}{RTT'}}$

For any single acid radical we have at the point of maximum buffer

at t^0

$$\overset{+}{H}_t = k'_a$$

and at t°

$$\frac{+}{H_2'} = \phi k'_a$$

From which by division

$$\frac{\frac{+}{H_2'}}{\frac{+}{H_2}} = \phi$$

or in logarithmic notation

$$pH_2 - pH_2' = \log \phi \quad (11)$$

TABLE II.

Calculation of the Heat of Ionization of Hemoglobin-Acid from the Total Titration Curves of Whole Blood at 38 and 15°C.

t	BHb	pH	$\log \phi$	Heat of ionization of one acid radical of hemoglobin.
$^{\circ}\text{C.}$	<i>mols</i>			<i>cal.</i>
38	2.0	7.21	0.56	-10,000
15	2.0	7.77		

TABLE III.

Shift of pH of Maximum Buffer per 1.0°C. at 38°C. for Any Acid Radical with a Negative Heat of Ionization.

q_{ac}	Example.	ϕ	$\frac{\Delta pH}{1^{\circ}\text{C.}}$ of maximum buffer.
<i>cal.</i>			
-13,000	Amino acids.	1.07	-0.0292
-10,000	Hemoglobin-acid.	1.05	-0.0224
-2,800	Carbonic acid.	1.01	-0.0063

Table III shows the change of pH of maximum buffer per 1°C. for acids with varying heats of ionization calculated from equation (11).

The two acids of importance as buffers in the blood are carbonic acid and hemoglobin. The heat of ionization of carbonic acid is -2,800 cal. and that of hemoglobin is -10,000 cal. It is evident that a rise in temperature has an appreciable effect on their buffer properties. This will be discussed later.

4. *Change of Total Buffer with Temperature.*—A consideration of Fig. 8 of Van Slyke's (1922) paper will make it clear that a shift of each buffer curve to the right or left will have no influence on the total buffer. Moreover, if we express the total molecular buffer of hemoglobin as

$$\beta_M = \frac{1}{C} \sum \frac{dB}{dpH} = \frac{2.3}{C} \left([B_{a1}] [H_a] + [B_{a2}] [H_{a2}] + \dots \right)$$

it is at once evident that since the expression is free of k_{a1}, k_{a2}, \dots its value is independent of temperature and dependent only on the concentration of the buffer.

The conclusion is reached that for reduced hemoglobin

$$\beta_M = \text{constant}$$

at varying temperature. Experimentally this may be shown from Fig. 1. The slope of the reduced curve gives the molecular buffer. The results are shown in Table IV.

TABLE IV.

Constancy of Molecular Buffer of Whole Blood of Hemoglobin at Varying Temperature.

<i>t</i>	β_M
°C.	<i>mols</i>
15	2.80
38	2.80

5. *Variation of the Excess of Base Bound by Oxyhemoglobin over Reduced Hemoglobin ΔB_{O-R} with Temperature.*—The increased base bound by completely or partially oxidized hemoglobin over completely reduced hemoglobin at constant pH is due to the increase of ionization of one acid radical whose pk' is changed from pk' to pk'_r .

For any given blood or hemoglobin solution at a temperature t let C = concentration of total hemoglobin which may be expressed in volumes per cent of oxygen, grams, or mols.

$(\Delta b)_{pH}$ = excess of base bound by the blood at any given fraction, x of oxygen saturation over the base bound when completely reduced. The pH is constant. $BHbO_2$ = mol fraction of the total oxidized "oxylabile" hemoglobin-acid group present at x .

BHb = mol fraction of the total *reduced* "oxylabile" hemoglobin-acid group present as *salt*.

Then it is apparent that

$$(x\text{CBHbO}_2 + (1-x)\text{BHbC})$$

is the amount of base bound to the "oxylabile" group when the blood is partially saturated and

$$(\text{CBHb})$$

is the amount of base bound by this acid group in the completely reduced state. At once we obtain

$$\begin{aligned} (\Delta b)_{\text{pH}} &= x\text{CBHbO}_2 + (1-x)\text{BHbC} - \text{CBHb} \\ &= C(\text{BHbO}_2 - \text{BHb})x \\ &\quad \left(\frac{k'_{\text{ot}}}{k'_{\text{ot}} + \text{H}} - \frac{k'_{\text{rt}}}{k'_{\text{rt}} + \text{H}} \right) \end{aligned} \quad (13)$$

If $C = 1$ mol and $x = 1$ (complete saturation) equation (13) may be put in the form

$$(\Delta\text{Bo-R})_{\text{pH}} = \frac{k'_{\text{ot}}}{k'_{\text{ot}} + \text{H}} - \frac{k'_{\text{rt}}}{k'_{\text{rt}} + \text{H}} \quad (13a)$$

where $(\Delta\text{Bo-R})_{\text{pH}}$ is the molecular increase of base bound at constant pH when 1 mol of hemoglobin combines with 1 mol of oxygen. Quite evidently it is a constant dependent only on the nature of the hemoglobin, the hydrogen ion concentration, and the temperature.

Differentiating equation (13) with respect to x we have

$$\begin{aligned} \left(\frac{\partial \Delta b}{\partial x} \right)_{\text{pH}} &= C \left(\frac{k'_{\text{ot}}}{k'_{\text{ot}} + \text{H}} - \frac{k'_{\text{rt}}}{k'_{\text{rt}} + \text{H}} \right) \\ &= \text{constant} \end{aligned}$$

Van Slyke, Hastings, and Neill (1922) have shown the constancy of $\left(\frac{\partial \Delta b}{\partial x} \right)_{\text{pH}}$ for various degrees of saturation in horse blood and hemoglobin solutions.⁴

⁴Van Slyke used the expression $\frac{dB}{dO_2}$ to correspond to $(\Delta b)_{\text{pH}}$ as here defined. This he found to be a linear function of the percentage saturation. Strictly speaking the expression $\left(\frac{\partial \Delta b}{\partial O_2} \right)_{\text{pH}}$ or what is the same thing $\left(\frac{\partial \Delta b}{\partial x} \right)_{\text{pH}}$ could be reserved for the rate of change of Δb with x . This is a constant.

For any other temperature t' similarly

$$\Delta B_{O-R} = [(BHbO_2)_{t'} - (BHb)_{t'}] \cdot \left[\frac{\frac{\phi k'_{ot}}{+}}{\phi k'_{ot} + H} - \frac{\frac{\phi k'_{rt}}{+}}{\phi k'_{rt} + H} \right] \quad (14)$$

where as before

$$\phi = \frac{k_{ot}}{k_{ot}'} = e^{\frac{q_{so}(T_1 - T_2)}{RT_1 T_2}}$$

The nature of the ΔB_{O-R} curve and the effect of temperature upon it is best shown in a general equation.

For simplicity denote the ratio of the dissociation constants of the oxylabile acid group by ϵ ; i.e.,

$$\frac{k'_{o'}}{k'_{r'}} = \epsilon \quad (15)$$

or in logarithmic form since $\log \frac{1}{k} = pk$

$$pk'_{r'} - pk'_{o'} = \log \epsilon \quad (16)$$

Then

$$\Delta B_{O-R} = \frac{\frac{k'_{o'}}{H} (\epsilon - 1)}{\left(\frac{k'_{o'}}{H} + 1 \right) \left(\frac{k'_{o'}}{H} + \epsilon \right)} \quad (17)$$

for another temperature

$$\Delta B_{O-R} = \frac{\frac{\phi k'_{o'}}{H} (\epsilon - 1)}{\left(\frac{\phi k'_{o'}}{H} + 1 \right) \left(\frac{\phi k'_{o'}}{H} + \epsilon \right)} \quad (18)$$

From these equations the general curves of ΔB_{O-R} in terms of mols of base per mol of hemoglobin, pH expressed as $pH - pk'_{o'}$ are shown in Fig. 2.

All these curves show the following general characteristics.

(1) Each curve is symmetrical and has two limbs asymptotic to a line, the zero ordinate. This zero ordinate is, of course, the titration curve of whole reduced blood and is represented by the equation

$$\beta_R = \beta_R (pH - pI) C \quad (19)$$

where β_R is the molecular buffer of reduced hemoglobin, pI the (or practically) isoelectric point of reduced hemoglobin, and C = the concentration of hemoglobin

(2) Each curve has a maximum which occurs at that point ($pH - pk'_o$) where the first derivative of equation (17) is 0; i.e.,

$$\frac{d}{dpH} \Delta B_{O-R} = \frac{\frac{k'_o}{H} (\epsilon - 1) \left\{ \left[\left(\frac{k'_o}{H} + 1 \right) \left(\frac{k'_o}{H} + \epsilon \right) \right] - \left[\frac{k'_o}{H} \left(\frac{k'_o}{H} + \epsilon \right) + \frac{k'_o}{H} \left(\frac{k'_o}{H} + 1 \right) \right] \right\}}{\left(\frac{k'_o}{H} + 1 \right)^2 \left(\frac{k'_o}{H} + \epsilon \right)^2}$$

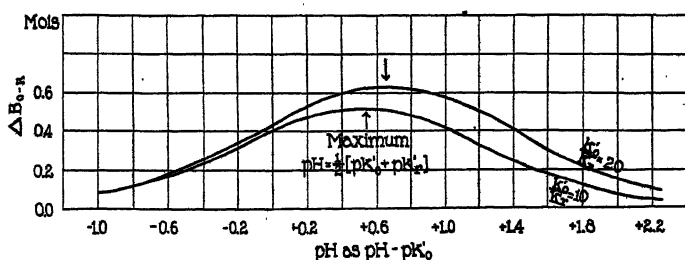


FIG. 2. General ΔB_{O-R} curve showing the effect of varying $\frac{k'_o}{k'_r} = \epsilon$.

This occurs when $\left(\frac{k'_o}{H} \right)^2 = \epsilon$

or

$$pH = \frac{1}{2} [pk'_o + pk'_r] \quad (20)$$

(3) At this point the absolute value of this maximum is found by substituting

$$\begin{aligned} \frac{k'_o}{H} &= \sqrt{\epsilon} \text{ in equation (17)} \\ \Delta B_{O-R}_{\max} &= \frac{\sqrt{\epsilon} (\epsilon - 1)}{(\sqrt{\epsilon} + 1) (\sqrt{\epsilon} + \epsilon)} \\ &= \frac{\sqrt{\epsilon} - 1}{\sqrt{\epsilon} + 1} \quad (21) \end{aligned}$$

which is independent of temperature.

results since this acid is as strong as H_2CO_3 , always in the evolution of CO_2 equal to $\frac{1}{n \text{ th}}$ of the oxygen taken up. This is untenable for several reasons: (1) The value of $\Delta \text{B}_{\text{O-R}}$ is not constant as has been shown here theoretically and experimentally. This had previously been shown experimentally by Van Slyke, Hastings, and Neill⁵ who also found that the $\Delta \text{B}_{\text{O-R}}$ values for horse blood were practically the same in whole blood and dialyzed hemoglobin over a wide range of pH. It is difficult to see why dialysis, which is known to produce great changes in the form of the oxygen dissociation curve and hence presumably according to the aggregation hypothesis, great changes in " n ," would be completely ineffective in altering a molecule of the form $\text{H}(\text{HbO}_2)_n$. If it were altered the values of $\frac{d(\Delta \text{B})_{\text{O-R}}}{dpH}$ in dialyzed hemoglobin would be markedly increased over the values in whole blood.

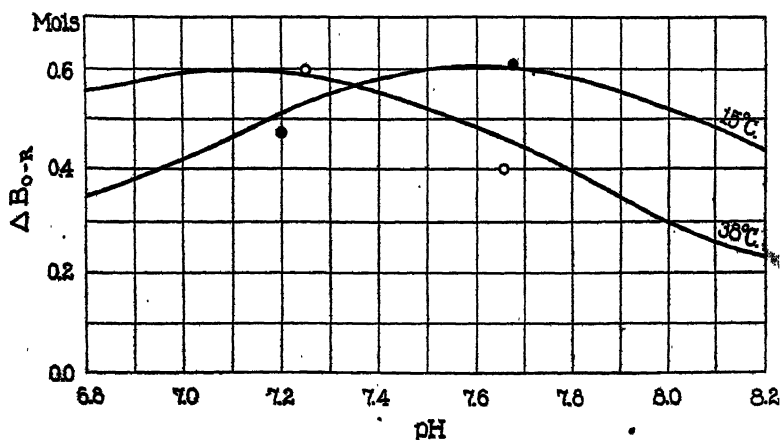


FIG. 3. $\Delta \text{B}_{\text{O-R}}$ curves at 38 and 15°C., from Table V.

6. *Variation of the Isoelectric Point of Hemoglobin with Temperature* $\frac{dpI}{dT}$.—For an amphoteric electrolyte the isoelectric point is

$$\text{H}^+ = \sqrt{\frac{k'_{\text{acid}}}{k'_{\text{base}}}} k_{\text{H}_2\text{O}}$$

⁵ Personal communication.

from which

$$pI = \frac{1}{2} (pk'_{acid} + pk'_{H_2O} - pk'_{base}) \quad (23)$$

Differentiating with respect to T

$$\begin{aligned} \frac{dpI}{dT} &= \frac{1}{2} \left[\frac{d(pk'_{acid})}{dT} + \frac{d(pk'_{H_2O})}{dT} - \frac{d(pk'_{base})}{dT} \right] \\ &= \frac{-1}{2RT^2} (q_{acid} + q_{H_2O} - q_{base}) \end{aligned} \quad (23a)$$

since

$$RT^2 \frac{d \ln k'}{dT} = q_{ac} = q_{H_2O} = q_{base}$$

the respective heats of ionization.

Upon integration of equation (23 a) we have in 10 base logs

$$pI - pI' = \Delta pI = \frac{-(T - T')}{2.3 \cdot 2 R T T'} (q_{acid} + q_{H_2O} - q_{base}) \quad (24)$$

which gives the approximate relation of the change of isoelectric point of hemoglobin (or other protein) to the temperature.

7. *Calculation of the Heat of Ionization of Hemoglobin as Base per Equivalent OH⁻.*—Practically, as Levine (1923) has shown, for a weak polybasic acid of the nature of hemoglobin the isoelectric point is a function of the minimum pk_{acid} and pk_{base} . Equation (24), then, may be used to calculate the heat of ionization of the basic radicals of hemoglobin from the shift of the isoelectric point with temperature, if as for the acid radicals we assume identical heats of ionization for the basic radicals. We tentatively assume that the heat of ionization of the basic radicals is of the same order of magnitude as that of the acid radicals, since Meyerhof (1922) has shown this for other protein. We calculate from equation (24) that ΔpI from 38–20° is -0.28 pH. This is in complete accord with the experimental data quoted by Warburg (1922)* who gives the shift of the isoelectric point from 38° to room temperature as -0.3 . From this agreement we conclude that the heat of ionization of hemoglobin as a base is of the same order or magnitude as that acid; viz., $-11,000$ to $-13,000$ cal.

* Warburg (1922), p. 298.

8. *Effect of Oxygenation on the Basic Properties of Hemoglobin. Calculation of ΔpI of Oxy- and Reduced Hemoglobin.*—Of all inorganic and organic compounds known, hemoglobin is unique in that it is capable of combining with oxygen in all proportions up to a definite stoichiometric limit and of establishing equilibria with oxygen at variable fractions of this limit. Added to this is the peculiar property through which by virtue of oxidation it increases on the alkaline side of the isoelectric point the base-binding properties of one moiety of the molecule thereby becoming an unusually effective buffer in a system constantly subjected to acid-base shifts.

It is conceivable since hemoglobin is an ampholyte that oxidation might increase the acid-binding properties below the isoelectric point. That the acid-base lability is confined to the acid portion of hemoglobin is rendered highly probable by the following. From equation (23) we obtain for reduced hemoglobin at constant temperature

$$pI_{(r)} = \frac{1}{2} [pk'_{acid(r)} + pk'_{H_2O} - pk'_{base(r)}]$$

and for oxyhemoglobin

$$pI_{(o)} = \frac{1}{2} [pk'_{acid(o)} + pk'_{H_2O} - pk'_{base(o)}]$$

From which by subtraction

$$pI_{(r)} - pI_{(o)} = \Delta pI_{O-R} = \frac{1}{2} (\Delta pk'_{acid} - \Delta pk'_{base}) \quad (25)$$

From this equation it is manifest that if oxidation of hemoglobin changed the basic dissociation of one basic radical as much as it does the acid dissociation of the one "oxylabile" acid radical, there would be no shift of pI on oxidation. It is known, however, that it is shifted and from the values of

$$\log \frac{k_o}{k_r} = pk_r - pk_o = \Delta pk_{acid} = 1.0$$

we would expect a shift of 0.5 in pI upon oxidation of hemoglobin. In point of fact the value found experimentally is somewhat lower. But the remaining acid groups not affected by oxidation would tend partially to nullify this change in pI produced by oxidation. We would expect the actual ΔpI to be less than calculated. The fact that it changes at all is strongly in favor of the hypothesis that there is no increase in the basic ionization of hemoglobin

corresponding to the acidic increase upon oxidation. The evidence is as yet too inconclusive to warrant more than a statement of the hypotheses which is now being tested in this laboratory.

9. *Significance of Change of the Buffer Curves of Carbonic Acid and Hemoglobin in Fever.*—A rise of 3°C. in infectious diseases such as pneumonia is by no means rare. A consideration of Fig. 4 shows the significance of these changes on the acid-base equilibrium. At 41°C. the total titration curve of hemoglobin plotted in terms of

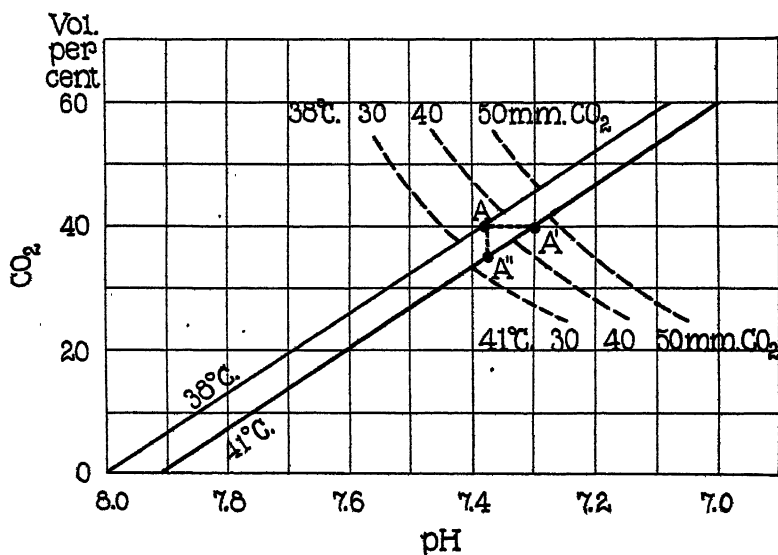


FIG. 4. Effect of fever on the CO_2 absorption curve.

BHCO_3 is parallel to the curve at 38°C. But from Table II there is a shift of 0.0224 pH per 1°C. or about 0.09 pH to the acid side.

At 38°C.

$$\text{pk}_{\text{H}_2\text{CO}_3} = 6.15$$

and

$$\frac{\alpha_{\text{CO}_2}}{760} = 0.0672$$

For a normal individual we may say for illustration that for the point "A" of arterial blood we have

$$\begin{aligned} \text{H}_2\text{CO}_3 + \text{BHCO}_3 &= 40 \text{ vol. per cent} \\ \text{pH} &= 7.37 \\ \text{CO}_2 \text{ tension} &= 36 \text{ mm.} \end{aligned}$$

If the available base (alkaline reserve) be unchanged we would have if the blood carried the same load of carbonic acid ($\text{BHCO}_3 + \text{H}_2\text{CO}_3$) at $t = 41^\circ\text{C}$.
when

$$\text{pk}_{\text{H}_2\text{CO}_3} = 6.13$$

and

$$\frac{\alpha_{\text{CO}_2}}{760} = 0.0626$$

at the point A'

$$\begin{aligned} \text{H}_2\text{CO}_3 + \text{BHCO}_3 &= 40 \text{ vol. per cent} \\ \text{pH} &= 7.28 \\ \text{CO}_2 \text{ tension} &= 45 \text{ mm.} \end{aligned}$$

If, however, the pH remained the same then at 41°C . for the point A''

$$\begin{aligned} \text{H}_2\text{CO}_3 + \text{BHCO}_3 &= 37 \text{ vol. per cent} \\ \text{pH} &= 7.37 \\ \text{CO}_2 \text{ tension} &= 34 \text{ mm.} \end{aligned}$$

In other words, without any change in the total alkali reserve fever results if uncompensated by increased H_2CO_3 excretion (respiratory compensation) in an increase of the acidity of the blood and an elevation of the CO_2 tension (alveolar CO_2). For maintenance of constant pH the lungs must compensate by extra excretion of CO_2 (dyspnea) to a point 3 to 4 volumes below the normal in total CO_2 and 2 to 3 mm. of Hg below for alveolar CO_2 .

A subject immersed in a hot bath has fever with a minimum of complicating factors. In heat dyspnea Haggard (1920) found that a subject immersed in a hot bath and having a temperature of $106\text{--}107^\circ\text{F}$. has a lowering of the bicarbonate of the blood, lowering of the alveolar CO_2 , and an increased C_H , all quite in accord with the above theoretical predictions. Incidentally it is evident that a lowering of the BHCO_3 of blood or plasma in fever is by no means an indication of the diminution of the total available base at elevated temperatures unless this diminution is in excess of 3 to 4 volumes per cent.

In disease accompanied by fever many complicating factors come into play, making conclusions more difficult. There is sufficient evidence, however, to show that in pneumonia there is a general tendency toward changes in the direction indicated above. For example, the work of Hastings and Binger (1923) shows that in pneumonia there is slight but definite decrease in arterial pH, and CO_2 tension, while the work of Means, Boothby, Peabody (1912), and others shows that the bicarbonate in the blood and alveolar CO_2 is low.⁷

10. *Correction of Arterial pH Points in Fever.*—The pH of the arterial blood of patients is frequently determined by plotting the CO_2 absorption curve at 38°C . and from the known arterial CO_2 content reading off the arterial pH on CO_2 -pH diagram. When the subject has a higher temperature than 38°C . a correction must be made as is evident from the above discussion.

The shift of the CO_2 absorption curve at 38°C . is (Table III)

$$\Delta\text{pH} = \log \varphi = \frac{q_{\infty}}{2.3 RT^2} = -0.022 \text{ pH per } 1^\circ\text{C. which is the correct factor to be employed; i.e.,}$$

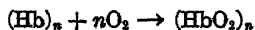
$$\text{pH}_t = \text{pH}_{38} - 0.022 (t - 38)$$

An example will make this clear. The patient's whole blood CO_2 -pH curve at 38° has been determined from two (or one, Peters, Bulger, and Eisenman, 1924) points. Arterial blood has 32.0 volumes per cent CO_2 . This gives pH 7.50 at 38°C . Patient's temperature = 41° .

$$\text{Corrected pH at } 41^\circ\text{C. } 7.50 - 0.022 \times 3 = 7.43$$

Part II. Thermodynamic Relations of the Oxygen-Binding Properties of Whole Blood.

1. *Thermodynamic Derivation of the Relation of K_O to k'_o , k'_r , and \bar{H}^\dagger .*—It may be shown thermodynamically without assumption as to the kinetics of the reaction



that K_O , the oxygen dissociation constant, is a function of k'_o , k'_r , and \bar{H}^\dagger . The basic assumption made is that oxygen combines

⁷ It is by no means intended to assert that in fever in disease the above mechanism is the sole or even the chief factor in these changes.

with hemoglobin at one place only within the molecule at, or in close proximity to, an acid radical whose ionization is thereby increased. The weight of chemical evidence definitely warrants this assumption.

On general thermodynamic grounds the following is true. If the combination of oxygen with this oxylabile acid group increases its ionization (*i.e.* increases at any pH its salt) in the ratio

$$\frac{k'_o}{k'_r}$$

then the affinity of oxygen for the ion (and likewise for the salt) is increased in the same ratio; *i.e.*,

$$\frac{K_s}{K_a} = \frac{k'_o}{k'_r} \quad (26)$$

and

$$K_s : K_a : K_i$$

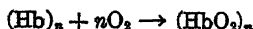
where K_s , K_a , K_i are the oxygen equilibrium (or dissociation) constants for the salt, acid, and ionic form of hemoglobin, respectively.

If this were not true there is at present no conceivable physical-chemical explanation for the variation of the affinity of oxygen for hemoglobin with changing \bar{H}^+ . This general principle allows us to equate the affinity of oxygen for hemoglobin in terms of the acid group involved.

The free energy change of any true chemical reversible reaction is given by the van't Hoff isotherm

$$-\Delta F = \log K - \sum \nu \log C \quad (27)$$

where K is the equilibrium constant of the reaction concerned and $\sum \nu \log C$ is the summation of the logs of the concentrations of the *resultants* over the concentration of the *reactants* raised to required powers. For the reaction



we have if the reaction goes completely from left to right

$$-\Delta F = RT \log K_o - RT \log \frac{(\text{HbO}_2)}{(\text{Hb}) (\text{O}_2)^n}$$

Since

$$(\text{HbO})_2 = 1 \text{ mol}$$

$$(\text{Hb}) = 1 \text{ mol}$$

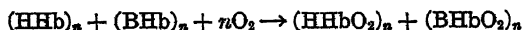
$$(\text{O}_2) = 1 \text{ mol}$$

we have

$$-\Delta F = \log K_O \quad (28)$$

where as before K_O is Hill's oxygen dissociation constant.

Consider the reaction as occurring at constant pH between salt of reduced hemoglobin and acid of reduced hemoglobin producing salt and acid of oxyhemoglobin according to the equation



The "oxylabile" acid group only is concerned, the rest of the molecule remains unaltered.

From the van't Hoff isotherm, substituting for $\Sigma \nu C$ the concentration of the *resultants* over the reactants, we write

$$-\Delta F = RT \log K'_A - RT \log \frac{\frac{k'_o}{k'_o + \overset{+}{\text{H}}} \cdot \frac{\overset{+}{\text{H}}}{k'_o + \overset{+}{\text{H}}}}{\left(\frac{k'_r}{K'_r + \overset{+}{\text{H}}}\right) \cdot \left(\frac{\overset{+}{\text{H}}}{K'_r + \overset{+}{\text{H}}}\right) \cdot (\text{O}_2)^n} \quad (29)$$

From equations (28) and (29) since $(\text{O}_2) = 1$

$$K_O = K_A \frac{(k_o + \overset{+}{\text{H}})^2}{(k_r + \overset{+}{\text{H}})^2} \quad (30)$$

where K_A is the absolute oxygen affinity or equilibrium constant of hemoglobin independent of $\overset{+}{\text{H}}$.

$$* \quad \frac{k'}{k' + \overset{+}{\text{H}}} = \text{concentration of salt}$$

$$1 - \frac{k'}{k' + \overset{+}{\text{H}}} = \frac{\overset{+}{\text{H}}}{k_o + \overset{+}{\text{H}}} = \text{concentration of acid}$$

This expression is derived independently of any assumptions regarding the kinetics of the reaction, the form of the molecule, or the value of "n." It will be shown to be consistent with our experimental knowledge of the dependence of K_O on \bar{H}^+ as well as k'_o and k'_r . Equation (30) may be transformed into a general form as before at constant temperature

$$\frac{k'_o}{k'_r} = \epsilon; \log \epsilon = pk'_r - pk'_o.$$

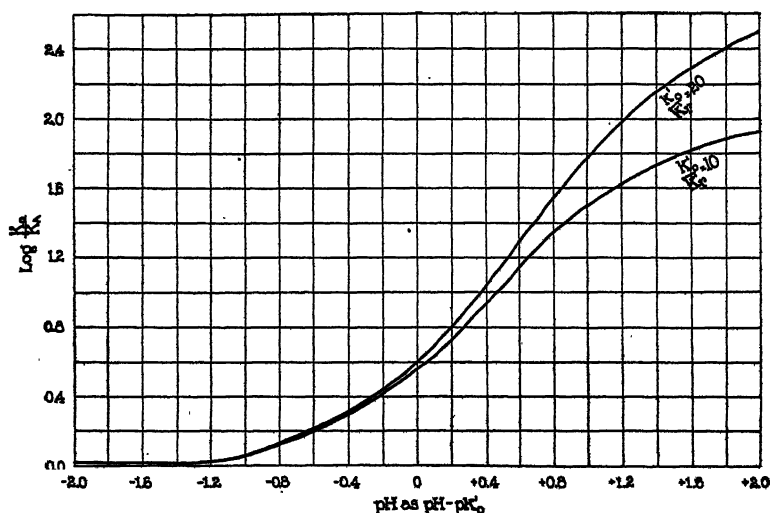


FIG. 5. General P-B lines showing the effect of varying $\frac{k'_o}{k'_r} = \epsilon$.

then.

$$\log K_O = \log K_A + \log \left[\frac{\epsilon \left(\frac{k'_o}{H} + 1 \right)}{\left(\frac{k'_o}{H} + \epsilon \right)} \right] \quad (31)$$

From equation (31) we may construct "general" P-B lines.

These general P-B lines (Fig. 5) have the following properties: (1) On the acid side they are asymptotic to a limiting value of $\frac{K_O}{K_A}$ which is the log of the equilibrium constant, K_a , of hemo-

TABLE V.
 ΔB_{O-R} at 15 and 38°C. on Whole Blood.

<i>t</i>	Oxygen capacity.	$\Delta CO_2 R - O$	pH	ΔB_{O-R}
°C.	vols. per cent	vols. per cent		mols.
15	19.0	9.2	7.20	0.48
15	19.0	11.4	7.68	0.60
38	19.0	10.5	7.25	0.60
38	19.0	8.0	7.66	0.42

See Fig. 3.

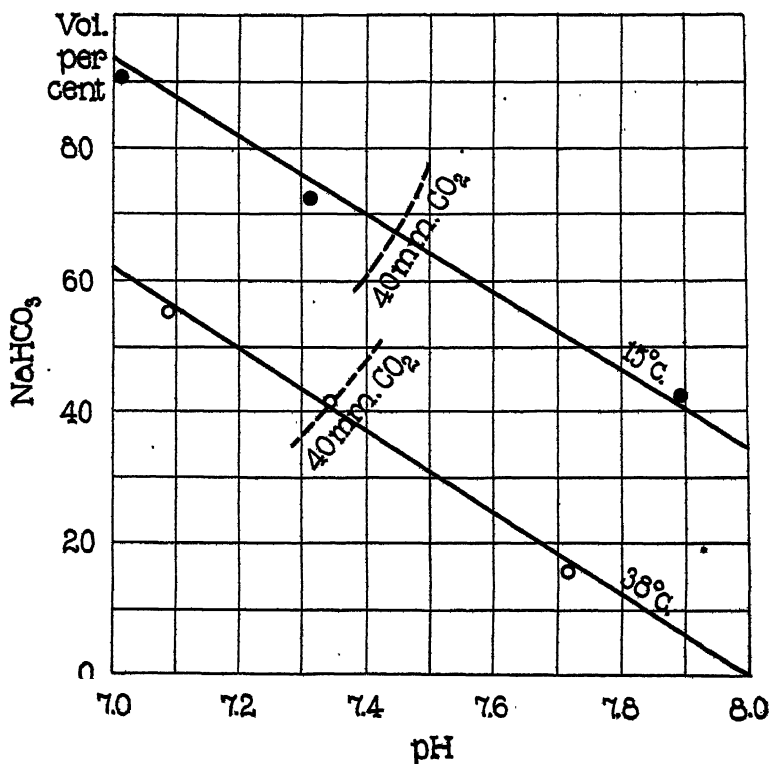


FIG. 6. CO₂ absorption curves of W. C. S. at 38 and 15°C. at approximately half saturation.

globin-acid and oxygen. (2) On the alkaline side the limiting asymptote is the log of the equilibrium constants K_s or K_i of hemoglobin salt or ion and oxygen. (3) The intermediate portion of the curve has a double curvature. Throughout the range of pH — pK_o (+ 0.5 to 1.5, which at 38° corresponds to pH 7 to 8) usually studied the curve is practically a straight line. The departure from straightness over this range is less than the present analytical method could detect so that the current conception that the P-B lines are straight holds only for this range of pH. (4) At constant temperature of pH log K_o is dependent on fixed constants of hemoglobin.

TABLE VI.

Comparison of Calculated and Observed K_o at 15 and 38°C.

$$k_o = 10^{-6.5} \quad k_r = 10^{-7.8}$$

pH	$\frac{K_o}{K_A} = \left(\frac{k'_o + H}{k'_r + H} \right)^2$ Calculated.	K_o observed.		$\frac{K_o \text{ (observed)}}{K_o \text{ (calculated)}}$	
		15°C.	38°C.	15°C.	38°C.
		$\times 10^4$	$\times 10^4$		
7.0	13.1	158	120	12.1	9.2
7.2	23.5	250	219	10.7	9.3
7.4	41.4	380	398	9.2	9.6
7.6	71.0	575	708	8.1	10.0
7.8	110.0	912	1,260	8.3	11.5
8.0	161.0	1,410	2,290	8.7	14.2

The relation given in equation (30) may be tested experimentally (from Experiments 4 and 5) by comparing the values of $\frac{K_o}{K_A}$ calculated from it with observed values at 15 and 38°C. given in Fig. 6. The comparison is given in Table VI. The values of k'_o and k'_r used are those calculated from Experiment 2. Since the scales of the calculated and observed constants differ it is necessary to compare their ratios which are given in the last column. For each temperature the constancy is satisfactory.

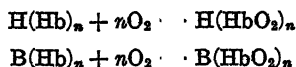
Recently, two other relations of K_o in terms of k'_o , k'_r , and $\frac{1}{H}$ have appeared in the literature. The first is by Henderson (1920)³ and in the terminology of the present paper is

³ Henderson (1920), p. 414.

$$K_O = \text{constant} \frac{k'_r + \frac{+}{H}}{(k'_r)^2 + k'_o H + \frac{+}{H}}$$

The method of derivation of this expression is not given.

The second is derived by Hill (1923) from the kinetics of the reactions.



This involves an unwarranted assumption concerning the form of the hemoglobin molecule which has been previously discussed. The relation in the symbols used here is

$$K_O = \text{constant} \frac{k'_r + \frac{+}{H}}{k'_r + \frac{+}{H}}$$

which is somewhat similar to the expression derived here.

In order to compare these three expressions with experiment use is made of Barcroft's (1914) data given K_O at varying C_H .

Table VII shows K_O calculated from each expression compared as before by the ratios of the calculated K_O to the observed K_O for Barcroft's blood at 37°C.

It is evident that the first two expressions for K_O give calculated values which are widely divergent from the experimental values. On the other hand, the expression derived here thermodynamically gives values of K_O and which agree practically with those obtained on the blood of Barcroft at 37° and for the blood of W. C. S. at 15 and 38°C.

2. *Alterations of P-B Lines in Disease.*—Equation (31) shows that $\log K_O$ is dependent only upon fixed constants for hemoglobin and relative hydrogen ion concentrations. Therefore, only two conditions in disease can bring about alterations in the slope or position of the P-B lines; *viz.*, (1) changes in temperature of the blood producing relative changes in k'_o and k'_r , or (2) absolute changes in the constants pk'_o and pk'_r , produced by changes in the nature of hemoglobin. Since K_O values are usually obtained *in vitro* at 38° regardless of the patient's temperature the first condition is of no significance. The latter is extremely un-

likely, and in point of fact studies on the P-B lines in disease have given on the whole negative results in so far as any consistent changes in position and slope are concerned although variations from individual to individual have been observed. It is easy to see also from the above why the addition of lactic acid to whole blood as reported by Donegan and Parsons (1920) produced no change in the P-B line. Similarly, for hemoglobin in equilibrium

TABLE VII.

Comparison of the Henderson, Hill, and Thermodynamic Relation of K_O to

k'_o , k'_r , and H with Experimental K_O Values of Barcroft's Blood.
 $k_o = 10^{-6.5}$ $k_r = 10^{-7.8}$

C_H $\times 10^3$	K_O (Barcroft). $\times 10^3$	K_O (Henderson) = $f\left(\frac{k'_r + H'}{(k')^2_r + k'_o H}\right)$		K_O (Hill) = $f\left(\frac{k'_o + H}{k'_r + H}\right)$		$K_O =$ $f\left(\frac{k'_o + H^+}{k'_r + H}\right)^2$	
		Calculated.	$\frac{K_O \text{ (observed)}}{K_O \text{ (calculated)}}$	Calculated.	$\frac{K_O \text{ (observed)}}{K_O \text{ (calculated)}}$	Calculated.	$\frac{K_O \text{ (observed)}}{K_O \text{ (calculated)}}$
1	131.0	834	15.6	12.7	10.3	161.0	8.1
2	79.0	588	13.3	9.45	8.4	89.5	8.8
3	50.5	500	10.2	7.71	6.5	58.0	8.7
4	36.1	456	7.9	6.44	5.6	41.1	8.7
5	27.6	427	6.4	5.61	4.9	31.4	8.8
6	21.9	408	5.4	5.00	4.4	25.0	8.8
7	18.0	395	4.5	4.53	4.0	20.6	8.7
8	14.9	384	3.8	4.16	3.6	17.4	8.6
9	12.5	378	3.3	3.87	3.2	14.9	8.4
Ratio of change over about 1 pH unit.....		4.7		3.2		0	

with CO Barcroft and Murray (1922) found that HCl and CO₂ had precisely the same effect on the values of K_{CO} .

3. *Relation between Heat of Oxidation of Hemoglobin in Whole Blood at Constant pH and Constant CO₂ Tension.*—The heat of oxidation of whole blood is thermodynamically calculated from the relation $\left(\frac{\delta \ln K_O}{\delta T}\right)_{pH} = \frac{Q_O}{RT^2}$. In this paper the values of K_O at two temperatures have all been compared at the same pH.

It is obvious that since pH has such a marked influence on K_0 that this isohydric comparison is essential and moreover is self-evident since $\left(\frac{\delta \ln K_0}{\delta T}\right)_{\text{pH}}$ is a partial differential with pH constant. Barcroft, Hill, and others have failed to appreciate this point and have compared K_0 values at constant CO_2 tension.*

The relation between the CO_2 tensions at any two temperatures required to yield the same pH for whole blood may be derived from the Hasselbalch equation and a knowledge of the ratio NaHCO_3 concentration at constant pH for the two temperatures. Thus for a temperature t ,

$$\text{pH} = \text{pK}_1 + \log \frac{[\text{NaHCO}_3]}{760 \text{ } ^\text{p}\text{CO}_2}$$

and for t' ,

$$\text{pH} = \text{pK}'_1 + \log \frac{[\text{NaHCO}_3]'}{760 \text{ } ^\text{p}'\text{CO}_2}$$

For the same pH

$$\frac{^\text{p}\text{CO}_2}{^\text{p}'\text{CO}_2} = \frac{[\text{NaHCO}_3]}{[\text{NaHCO}_3]'} \cdot \frac{\alpha}{\alpha'} \cdot 10^{\text{pK}_1 - \text{pK}'_1}$$

For the blood of W. C. S. (Fig. 6) the ratio of $\frac{[\text{NaHCO}_3]}{[\text{NaHCO}_3]'}$ at 15 and 38°C . between pH 7.6 and 7.2 is 1.7 to 2.3.

Therefore

$$\frac{^\text{p}\text{CO}_2 \text{ } 15}{^\text{p}'\text{CO}_2 \text{ } 38} = 1.7 \text{ to } 2.3 \times \frac{0.56}{1.00} \times 10^{+6.3 - 6.15} = 1.3 \text{ to } 1.8$$

* Hill compares the K_0 values at 38° at 40 mm. of CO_2 with K_0 values at lower temperatures (say 15°C .) at CO_2 tensions which at 38° would equal 40 mm., in other words $40 \times \frac{275 + 15}{275 + 38} = 37$ mm. of CO_2 .

TABLE VIII.

Ratio of p_{CO_2} at 15 and 38°C. to Give Same pH for Average Whole Blood.

<i>t</i>	pH	NaHCO ₃	CO ₂	$\frac{P_{15}}{P_{38}}$
°C.		vols. per cent	mm.	
15	7.6	59	24	1.8
38	7.6	25	13	
15	7.2	84	87	1.3
38	7.2	50	66	

An example will make this clear. From Fig. 6, Experiments 4 and 5, we obtain the following.

The difference in the heat of oxidation of whole blood at constant CO₂ tension and constant pH may be calculated from the CO₂ absorption curves and the P-B lines shown in Figs. 6 and 7.

In Fig. 6 we have plotted the 40 mm. CO₂ point on both absorption curves since this is usually selected as the CO₂ tension for equilibrium. From the two charts the following data are collected.

Difference in the Heat of Absorption of O₂ by Whole Blood per n Mols of Hemoglobin, Q_O, when Calculated from K_O at Constant CO₂ Tension and K_O at Constant pH.

<i>t</i>	CO ₂	pH	$\log \frac{1}{K_O}$	Q _O at 40 mm. CO ₂	Q _O at pH 7.42
°C.	mm.			cal.	cal.
15	40*	7.45	2.38	19,500	17,100
38	40	7.34	3.48		
38		7.45	3.34		

*Hill's choice of 37 mm. of CO₂ pressure at 15°C. would increase Q_O by 500 cal.

By this method of calculation the value of Q_O is found in complete accord with the results of Barcroft, Hill, and others who have determined directly and from the equilibrium constants a value of 19,000 to 20,000 cal. At constant pH = 7.4 Q_O is 17,100.

SUMMARY AND CONCLUSIONS.

1. A résumé of the main facts concerning the oxygen and base-binding power of the whole blood is given with especial reference to the effect of temperature on these properties.

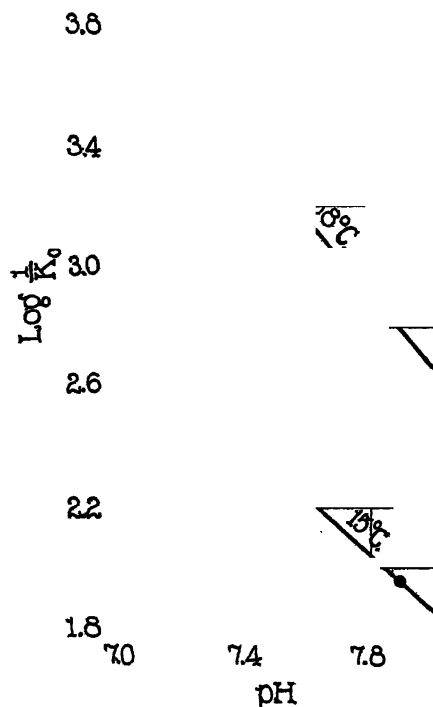


FIG. 7. P-B lines of W. C. S. at 38 and 15°C.

2. The value of $pK_{H_2CO_3}$ determined for whole blood at 15°C. shows that the van't Hoff isochore

$$\frac{d \ln K}{d T} = \frac{q}{RT^2}$$

where q is the heat of ionization and K the equilibrium constant, holds for H_2CO_3 in whole blood. The value of the heat of ionization of H_2CO_3 in whole blood thus calculated is practically the same as in aqueous solutions.

3. The heat of ionization of hemoglobin-acid per equivalent of H^+ was calculated thermodynamically by means of the van't Hoff isochore from the shift of the BHB-pH line of whole blood from 38 to 15°. The value obtained -10,000 cal. is in agreement with the directly determined heats of ionization of other proteins. The validity of the van't Hoff isochore for hemoglobin-acid in whole blood is thus established.

4. The heats of ionization of all the acid radicals of hemoglobin are practically the same. This follows because BHB-pH lines for whole blood are straight lines at both 15 and 38°C. and parallel.

5. The effect of temperature on the buffer value of whole blood is discussed. The molecular buffer of hemoglobin is theoretically independent of temperature. Experimentally it was found to be

$$\begin{aligned}\beta_M &= 15^\circ\text{C.} = 2.8 \text{ mols.} \\ &= 38^\circ\text{C.} = 2.8 \text{ "}\end{aligned}$$

6. The excess of base bound by oxyhemoglobin $(\Delta\text{BO-R})_{\text{pH}}$ over reduced hemoglobin is shown to be

$$(\Delta\text{BO-R})_{\text{pH}} = \frac{\frac{k'_o}{\text{H}^+}(\epsilon-1)}{\left(\frac{k'_o}{\text{H}^+} + 1\right)\left(\frac{k'_o}{\text{H}^+} + \epsilon\right)}$$

Curves were drawn from this expression which show the effect of k_o , k_r , $\frac{\text{H}^+}{\text{H}}$, and t upon $\Delta\text{BO-R}$. A method calculating k_o , k_r , and $\frac{dk_{\text{acid}}}{dT}$ from the above expression was indicated. For the blood of W. C. S. pk_o and pk_r were calculated to be 6.50 and 7.80 at 38°C.; i.e.,

$$\frac{k'_o}{k'_r} = 15 \pm 5$$

7. The relation of the shift of the isoelectric point of a protein with temperature to the heats of ionization of water and of the protein as acid and base is

$$\Delta\text{pI} = \frac{-(T - T')}{2.3 RTT'} (q_{\text{acid}} + q_{\text{H}_2\text{O}} - q_{\text{base}})$$

From this the heat of ionization of hemoglobin as base was calculated from the shift of the isoelectric point with temperature and

was found to be of about the same order of magnitude as the acid.

8. The relation between the isoelectric point of oxy- and reduced hemoglobin in terms of the change of the acid and basic dissociation constants upon oxidation is given as

$$\Delta pI_{O-R} = \frac{1}{2} (\Delta pK_{acid} - \Delta pK_{base})$$

From the known values of ΔpI_{O-R} and ΔpK_{acid} it was concluded that oxidation probably had no effect upon the basic properties of hemoglobin in contrast to its marked influence on the acid nature.

9. The magnitude of the heat of ionization of hemoglobin as an acid indicates that a small change in temperature produces a large change in the strength of hemoglobin as an acid.

10. The significance of the temperature effect on the base- and oxygen-binding properties of blood is discussed in relation to fever. The theoretical predictions are found to be consistent with the experimental data.

11. The correction factor for arterial pH of patients with fever when determined from the CO_2 absorption curve at $38^\circ C$. is shown to be -0.022 pH per $1^\circ C$.

12. On the assumption that O_2 binds itself to hemoglobin in one place only and thereby increases the acidic properties of only one acid radical a theoretical relation for Hill's oxygen dissociation constant for hemoglobin

$$K_O = \left(\frac{k_o + \overset{+}{H}}{k_r + \overset{+}{H}} \right)^2 K_A$$

was deduced thermodynamically from van't Hoff's isotherm. It was shown that this relation is in accord with the experimental K_O values at $38^\circ C$. and $15^\circ C$. The same assumption was found to be consistent with the ΔB_{O-R} values found at 38 and $15^\circ C$. at varying pH.

13. From the above relation a general expression for $\log K_O$ was deduced. General "P-B" lines are drawn and their properties discussed. These lines have a double curvature and are straight over only a short pH range. The effect of disease on the P-B lines was discussed.

14. The relation between the heat of absorption of oxygen by blood at constant pH and at constant CO_2 tension was shown. At about pH 7.4 the heat at constant CO_2 tension is 3,000 cal. greater than at constant pH.

Experiment 1.

Determination of $pK_{H_2CO_3}$ for whole blood at $15^\circ C. \pm 0.1$

Determination of BHB — pH line for whole reduced blood at $15^\circ C.$

Venous blood was equilibrated with varying tension of CO_2 and varying oxygen saturations. Blood and gas phase separated and analyzed. pH of plasma read with both standards and diluted plasma at $15^\circ C.$ 0.04 added to observed readings.

$$\text{Dissolved } H_2CO_3 = \frac{1.019 \times 0.915}{760} P_{CO_2}$$

pK_1 at 15° on whole blood.

a. Determination of pK_1 at $15^\circ C.$ for Whole Blood.

b. Determination of BHB-pH Line for Whole Reduced Blood at $15^\circ C.$

No.	Capacity.	Saturation.	CO ₂	CO ₂	pH calculated.	pH observed.	pK ₁	NaHCO ₃	BHB per mol Hb.	(BHB) _a per mol Hb.
	vols. per cent	per cent	mm.	vols. per cent				vols. per cent	mols	mols
1	18.3	73	36.6	69.5	7.46	7.56	6.40	65.0	1.64	1:21
2	19.5	100	35.7	67.7	7.45	7.24	(6.09)†	63.3	1.63	1:07
3	18.9	40	101.2	97.8	7.12	7.12	6.30	88.8	0.33	0.13
	18.9	52	24.1	60.4	8.09	7.97	6.18	53.0	2.22	1.97
	18.9	51	6.9	37.0	7.93	7.87	6.24	36.0	3.12	2.83
4	19.9	61	144.2	108.4	6.93	7.01	6.38	90.7	0.23	0.0
	19.9	60	57.6	79.7	7.31	7.25	6.24	72.6	1.13	0.79
	19.9	45	8.9	43.6	7.90	7.94	6.34	42.5	2.64	1.89
5	20.0*	100	87.4	89.8	7.17	7.11	6.24	78.1	0.85	0.34
	20.0	100	33.2	66.2	7.48	7.43	6.25	62.1	1.65	1.05
	20.0	100	19.0	53.5	7.64	7.55	6.21	51.2	2.19	1.59
	20.0	100	10.5	41.5	7.81	7.67	6.16	40.2	2.74	2.20
6	20.0	80	111.2	97.8	7.09	7.09	6.30	84.2	0.54	0.15
	20.0	83	43.6	70.4	7.40	7.39	6.29	64.1	1.55	1.07
	20.0	88	11.9	44.3	7.77	7.72	6.25	43.8	2.56	1.99

Mean.....6.27 \pm 0.055

* Assumed.

† Excluded from mean.

*Experiment 2.**Determination of BHb-pH Line for Whole Reduced Blood at 33°C.*

No.	Date.	Oxygen capacity.	Saturation.	CO ₂ tension.	Total CO ₂ .	BHCO ₂	pH calculated.	BHb per mol Hb.	(BHb) ₂ per mol Hb.
	1923	vols. per cent	per cent	mm.	vols. per cent	vols. per cent		mols	mols
1	Apr. 23	20.6	52	96.1	62.0	55.5	7.09	1.92	1.60
2		20.6	49	39.8	49.2	41.5	7.34	2.60	2.38
3		20.6	51	6.9	17.8	17.3	7.72	3.78	3.55
4	July 20	19.6	80	43.0	40.7	37.8	7.46	2.93	2.51
5		19.6	49	30.0	41.3	39.3	7.44	2.84	2.49
6		19.6	25	31.0	46.0	43.9	7.46	2.61	2.48
7	Aug. 7	20.0	100	94.1	59.8	53.4	7.08	2.08	1.48
8	" 8	20.0	100	12.5	25.0	24.1	7.61	3.54	3.06
9	May 14	18.3	100	43.6	46.7	43.8	7.33	2.80	2.23
10	" 4	20.4	100	38.1	43.8	41.2	7.36	2.64	2.10
11	Apr. 24	20.6	100	52.0	47.6	44.1	7.25	2.47	1.89

*Experiment 3.**Determination of Δ BO-R at 15 and 33°C.*

No.	t	CO ₂	Whole blood.			pH calculated.
			O ₂	CO ₂	NaHCO ₂	
	°C.	mm.	vols. per cent	vols. per cent	vols. per cent	
1 R.	15	80.0	0.0	91.0	81.2	7.22
1 O ₂	15	76.3	19.0	81.4	72.1	7.20
2 R	15	17.9	0.0	58.0	55.8	7.71
2 O ₂	15	16.9	19.0	42.2	47.1	7.66
3 R	38	59.9	0.0	54.0	50.0	7.26
3 O ₂	38	47.3	19.0	42.7	39.5	7.25
4 R	38	13.0	0.0	30.7	29.8	7.68
4 O ₂	38	11.0	19.0	24.7	23.9	7.63

Experiment 4.

Determination of $\log \frac{1}{K_O}$ ($n = 2.39$) at Varying pH at 15°C. June 21, 1923.

Venous blood of W. C. S. 0.2 per cent oxalated. Equilibrated at 15 \pm 0.1°C. Blood and gas phase separated and analyzed.

No.	Oxygen.				CO ₂		pH calculated.	$\log \frac{1}{K_O}$
	Capacity.	Tension.	Content.	Saturation.	Tension.	Content.		
	vols. per cent	mm. Hg	vols. per cent	per cent	mm. Hg	vols. per cent		
1	19.93	17.6	12.08	60.6	144.2	108.4	7.01	2.79
2	19.93	15.4	13.48	67.7	57.2	79.7	7.31	2.52
3	19.98	6.1	9.13	45.2	8.9	43.6	7.89	1.96

Experiment 5.

Determination of $\log \frac{1}{K_O}$ ($n = 2$) at Varying pH at: °C. Apr..

Venous blood of W. C. S. 0.2 per cent oxalated. Equilibrated at 38 \pm 0.1°C. Blood and gas phase separated and analyzed.

No.	Oxygen.				CO ₂		pH calculated.	$\log \frac{1}{K_O}$
	Capacity.	Tension.	Content.	Saturation.	Tension.	Content.		
	vols. per cent	mm. Hg	vols. per cent	per cent	mm. Hg	vols. per cent		
1	20.56	40.5	10.58	51.5	96.1	62.0	7.09	3.814
2	20.56	27.9	10.12	49.2	39.8	44.2	7.34	3.469
3	20.56	18.5	10.44	50.8	6.9	17.8	7.72	3.014

See Figs. 2 and 3.

I wish to take this opportunity to thank Drs. Austin and Cullen, of the University of Pennsylvania, who read this manuscript with great care and made many valuable suggestions.

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CORRECTION.

On page 759, Vol. LIX, No. 3, April, 1924, in the numerator of Equations 10 and 11, K should be omitted, so that Equation 10 would read

$$\frac{LVcB}{BK + L}$$

and Equation 11

$$\frac{LVcG}{BK + L}$$

THE BUFFER MECHANISM FOR THE CALCION CONCENTRATION AND THE DETERMINATION OF CALCION BUFFER VALUES.

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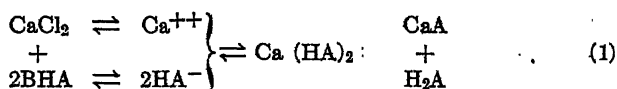
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An aqueous saturated solution of calcium chloride has a calcion concentration of about 5 mols per liter at 38°. A neutral saturated solution containing carbonates and phosphates at 38° has a calcion concentration of 0.0025 mol per liter (1). The calcion concentration of the first solution is two thousand times that of the second solution. These two solutions are analogous to an unbuffered and a buffered system of hydrions. The second solution is a buffered system in which additions of calcium salts do not increase

the calcion concentration proportionally to the amount added, nor does the removal of calcium salts decrease it proportionally. The calcion concentration is stabilized as the hydrion concentration is stabilized in buffered systems. The relations which determine the calcion concentration are the subject of this study.

The Nature of Calcion Buffers.

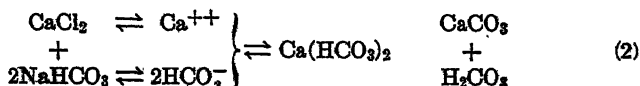
Addition of a highly dissociated calcium salt, *e.g.* CaCl_2 , produces a reaction in a mixture of a weak buffer acid, H_2A , and its primary salt, BHA, according to the equation,



The highly dissociated calcium salt combines with the buffer salt, BHA, to form the intermediate salt, $\text{Ca}(\text{HA})_2$. This calcium buffer salt is always in equilibrium with the least soluble, normal salt, CaA. The equilibrium is determined by the concentrations of buffer salt, BHA, and buffer acid, H_2A . Increase of concentration of calcium or buffer salt shifts the equilibrium from left to right; increase of concentration of buffer acid, from right to left. The calcion concentration is decreased in the first instance and is increased in the second instance. At equilibrium, calcion concentration is determined by the relative concentrations of total buffer salt, BHA plus $\text{Ca}(\text{HA})_2$, and free acid, H_2A . This constitutes a calcion buffer mechanism comparable to that for the hydrion buffer mechanism.

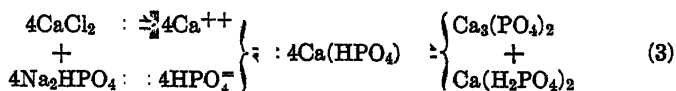
Calcion buffers are substances which resist the change in calcion concentration upon addition or removal of calcium salts. The calcion concentration is stabilized in the presence of mixtures of weak acids and their salts, which react to form insoluble normal calcium salts and soluble intermediate calcium salts.

The Carbonates as Calcion Buffers.—As an example of calcion buffering, the carbonates may be chosen. They react with a highly dissociated calcium salt, *e.g.* CaCl_2 , according to the equation,



where NaHCO_3 and H_2CO_3 are the buffer salt and acid, respectively; $\text{Ca}(\text{HCO}_3)_2$, the intermediate primary calcium salt; and CaCO_3 , the insoluble normal calcium salt. The calcion concentration of this system at equilibrium is determined by the ratio of the dissolved $\text{Ca}(\text{HCO}_3)_2$ and CaCO_3 , which is fixed by the ratio of concentrations of total buffer salt, NaHCO_3 plus $\text{Ca}(\text{HCO}_3)_2$, and free buffer acid, H_2CO_3 .

The Phosphates as Calcion Buffers.—The phosphates react in a similar manner with a highly dissociated calcium salt, *e.g.* CaCl_2 , according to the equation,



where Na_2HPO_4 and $\text{Ca}(\text{H}_2\text{PO}_4)_2$ are the buffer salt and buffer acid, respectively; CaHPO_4 , the intermediate secondary calcium salt; and $\text{Ca}_3(\text{PO}_4)_2$, the insoluble, normal calcium salt.

The Relations of Calcion Concentration in Terms of Calcion Buffers.

The Calcion Concentration in Terms of the Carbonates.—Experimental studies of the equilibrium relations of the carbonates in aqueous solution (1) have shown that at 38° , in the presence of solid CaCO_3 ,

$$\frac{[\text{Ca}^{++}] [\text{HCO}_3^-]^2}{[\text{H}_2\text{CO}_3]} = K_1 = 4.1 \times 10^{-5} \quad (4)$$

The HCO_3^- from carbonic acid in the concentration of carbonates equivalent to that present in blood is extremely small; *i.e.*, to the extent of about 1:10,000 of the H_2CO_3 . Essentially all the anion originates from the dissociation of the bicarbonates. Considering any soluble, monovalent, highly dissociable salt, BHCO_3 , as the source of HCO_3^- and assuming its average degree of ionizations to be γ , Equation 2 may be rewritten as

$$\text{Ca}^{++} = K_1 \frac{[\text{H}_2\text{CO}_3]}{[\text{HCO}_3^-]^2} \cdot \frac{K_1}{\gamma^2} \cdot \frac{[\text{H}_2\text{CO}_3]}{[\text{BHCO}_3]^2} \cdot K' \frac{[\text{H}_2\text{CO}_3]}{[\text{BHCO}_3]^2} \quad (5)$$

Inverting and expressing in logarithmic form,

$$pCa = pK' + \log \frac{[BHCO_3]^2}{[H_2CO_3]} \quad (6)$$

Where pCa and pK' are the negative logarithms of $[Ca^{++}]$ and K' , respectively.

The logarithmic unit, pCa , similar to the symbol pH , has been adopted for the numerical expression of the calcion concentration because it is more practical, more convenient to handle mathematically, and because changes in pCa are directly proportional to changes in pH . The physiological significance of this unit cannot be demonstrated because adequate data on the calcion concentration are not yet available.

TABLE I.
The Evaluation of the Carbonates as Calcium Buffers.

BHCO ₃	H ₂ CO ₃	pCa calculated from:	
		Determinations.	Equation 6.
<i>millimoles/l</i>	<i>millimoles/l</i>		
7.8	2.78	2.48	2.50
8.2	2.78	2.52	2.52
6.6	2.08	2.55	2.57
6.2	1.36	2.60	2.65
5.8	1.36	2.61	2.59
4.6	0.68	2.70	2.69
10.9	2.08	2.82	2.90
9.78	1.36	3.00	3.05
16.38	2.78	3.24	3.19
16.20	2.08	3.30	3.30
15.70	1.36	3.52	3.48
15.48	0.68	3.72	3.75

Equation 6 has been tested against the experimental data (1) given in Table I and can be interpolated from Fig. 1. Calculated from the relation,

$$pK' = \log \frac{K'}{(\gamma)^2}$$

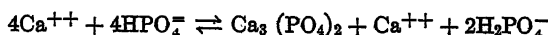
Where K' is 4.1×10^{-3} at 38° , and the average γ of $BHCO_3$ is 0.85, the value of the calcion pK' for the carbonates is 4.2.

The calcion buffering of the carbonates expressed by Equation 6 may be illustrated by two comparable experiments. Two aqueous systems at 38° contained in mols per liter:

Experiment.	CO ₂ tension.	H ₂ CO ₃	HCO ₃ ⁻	Ca ⁺⁺	pCa
	mm.				
1	20	0.00068	0.0038	0.00190	2.72
2	20	0.00068	0.0121	0.00019	3.72

Addition of 0.015 M NaHCO₃ to the calcium bicarbonate solution in Experiment 1 increased the HCO₃⁻ 3.1 times. The effect of this bicarbonate increase was a 10-fold decrease in the calcion concentration or a change in pCa from 2.72 to 3.72.

The Calcion Concentration in Terms of the Phosphates.—The relation for the calcion concentration in terms of the phosphates as calcion buffers may be developed similarly to the carbonates. The right-hand member of the buffering reaction 4, expressed ionically is,



The law of mass action gives,

$$\frac{[\text{Ca}^{++}]^4 [\text{HPO}_4^-]^4}{[\text{Ca}^{++}] [\text{H}_2\text{PO}_4^-]^2} = \frac{[\text{Ca}^{++}]^3 [\text{HPO}_4^-]^4}{[\text{H}_2\text{PO}_4^-]^2} = K_2 \quad (7)$$

Solving for Ca⁺⁺, we obtain,

$$\text{Ca}^{++} = K_2^{\frac{1}{3}} \cdot \frac{[\text{H}_2\text{PO}_4^-]^{\frac{2}{3}}}{[\text{HPO}_4^-]^{\frac{4}{3}}} \quad (8)$$

Considering the H₂PO₄⁻ as originating from any soluble, highly dissociable, monovalent, primary phosphate and assuming the average degree of ionization of the salt, BH₂PO₄, to be γ₁, and the HPO₄⁻ as originating from any soluble, highly dissociable, monovalent, secondary phosphate, B₂HPO₄, with an average degree of ionization γ₂,

$$\text{Ca}^{++} = \left(K_2^{\frac{1}{3}} \cdot \frac{(\gamma_1)^{\frac{2}{3}}}{(\gamma_2)^{\frac{4}{3}}} \right) \cdot \frac{[\text{BH}_2\text{PO}_4]^{\frac{2}{3}}}{[\text{B}_2\text{HPO}_4]^{\frac{4}{3}}} = K'' \cdot \frac{[\text{BH}_2\text{PO}_4]^{\frac{2}{3}}}{[\text{B}_2\text{HPO}_4]^{\frac{4}{3}}} \quad (9)$$

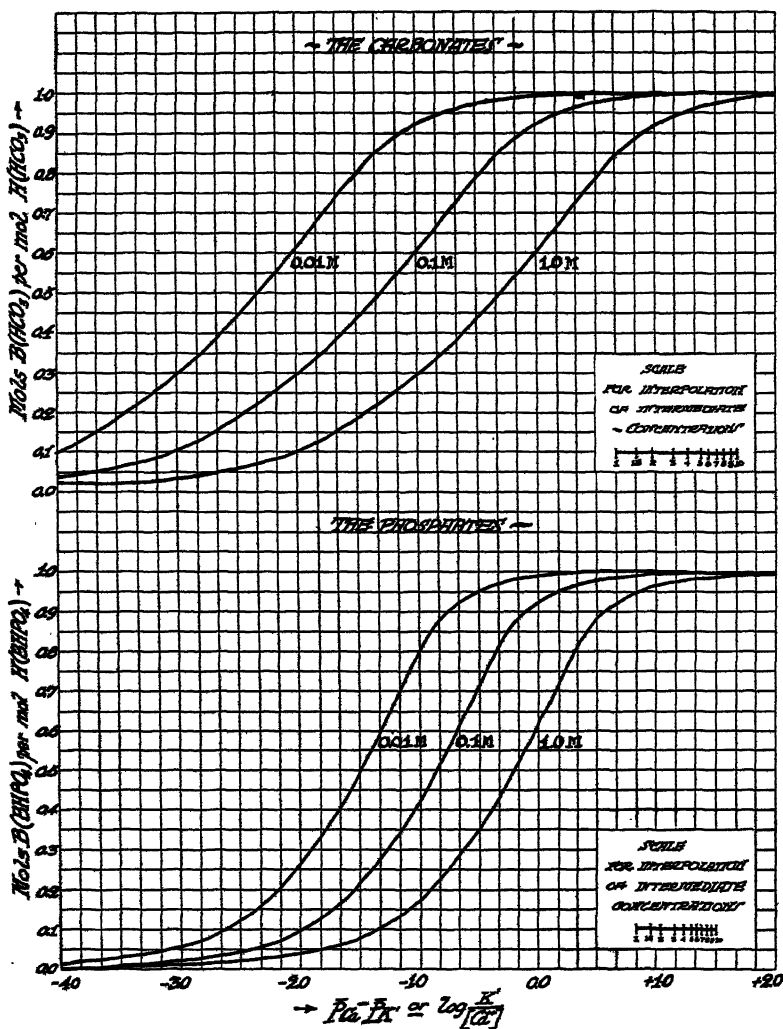


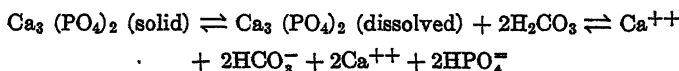
FIG. 1. Calcium buffer curves for the carbonates and phosphates, calculated from the equation, $pCa = pK + n \log \frac{[BA]^2}{[C] - [BA]}$.

Inverting and expressing in logarithmic form,

$$pCa = pK'' + \frac{2}{3} \log \frac{[B_2HPO_4]^2}{[BH_2PO_4]} \quad (10)$$

The curve representing this equation is given in Fig. 1. The value of pK'' and experimental data on the phosphates as calcion buffers will be reported in a separate paper.

In the presence of both carbonates and phosphates, the calcion concentration can be derived from either independently. Consider the reaction,



The law of mass action gives,

$$\frac{[Ca^{++}]^3 [HCO_3^-]^2 [HPO_4^{=}]^2}{[H_2CO_3]^2} = K_3 \quad (11)$$

since the concentration of dissolved $Ca_3(PO_4)_2$ in equilibrium with the solid phase is constant. From the first dissociation equilibrium of carbonic acid, we have,

$$\frac{[HCO_3^-]}{[H_2CO_3]} = \frac{K_4}{[H^+]}$$

Substituting in Equation 11,

$$\frac{[Ca^{++}]^3 [HPO_4^{=}]^2}{[H^+]^2} = \frac{K_3}{(K_4)^2} = K_5$$

Solving for Ca^{++} ,

$$Ca^{++} = K_5^{\frac{1}{3}} \cdot \frac{[H^+]^{\frac{2}{3}}}{[HPO_4^{=}]^{\frac{2}{3}}}$$

From the second dissociation equilibrium of phosphoric acid, we have,

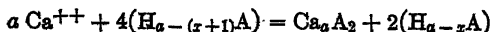
$$[H^+] = K_6 \frac{[H_2PO_4^-]}{[HPO_4^{=}]}$$

Substituting this equivalent of $[H^+]$ in the above equation,

$$Ca^{++} = K_7 \frac{[H_2PO_4^-]^{\frac{2}{3}}}{[HPO_4^{=}]^{\frac{2}{3}}}$$

which is identical with Equation 8.

The General Equation for the Calcion Concentration of Calcion Buffering Solutions.—Equations 3 and 4, expressing the buffering reactions of the carbonates and phosphates, are special forms of the general equation for any buffering solution,



where

x = an arbitrary valence number $< a$.

a = basicity of the acid; $a = x + 2$.

$H_{a-x}A$ = free buffer acid.

$H_{a-(x+1)}A$ = acid ion of the primary salt.

Ca_aA_2 = insoluble normal calcium salt of the buffer acid.

The law of mass action applied to the above equation gives,

$$\frac{[Ca^{++}]^a [H_{a-(x+1)}A]^4}{[H_{(a-x)}A]^2} = K$$

since the concentration of the dissolved Ca_aA_2 in equilibrium with the solid phase is constant. Solving for Ca^{++} and expressing the ionic forms whose valences are u and $u + 1$ respectively, in terms of their soluble, highly dissociable, monobasic salts,

$$Ca^{++} = K \cdot \frac{[B_u H_{a-x}A]^{\frac{2}{a}}}{[B_{u+1} H_{a-(x+1)}A]^{\frac{4}{a}}} \quad (12)$$

Inverting and expressing in logarithmic form,

$$pCa = pK + \frac{2}{a} \log \frac{[B_{u+1} H_{a-(x+1)}A]^2}{[B_u H_{a-x}A]} \quad (13)$$

The coefficient, $\frac{2}{a}$, is the ratio of the valence of calcium to the valence of acid, a . When the buffer acid, $[B_u H_{a-x}A]$, is free acid then u becomes zero, B_u disappears and the buffer acid is represented simply by $H_{a-x}A$. Equations 12 and 13 may be expressed more conveniently if we represent the concentration of free buffer acid by HA , the concentration of buffer salt by BA , and the valence ratio $\frac{2}{a}$, by n .

$$Ca^{++} = K \frac{[HA]^n}{[BA]^{2n}} \quad (14)$$

and

$$pCa = pK + n \log \frac{[BA]^2}{[HA]} \quad (15)$$

The calcium concentration is, therefore, dependent upon the ratio of the square of the salt concentration to the acid concentration.

This equation is represented graphically in Fig. 1. Equations 5, 6, 8, and 10 for the carbonates and phosphates are clearly special cases of the general equations Nos. 14 and 15, expressing the calcion concentration in terms of any buffering polyvalent acid and its soluble salt.

The Unit for Evaluation of Calcion Buffers.

The measure of the stability of the calcion concentration or the calcion buffer value can be derived from the relations which govern the calcion concentrations in terms of its buffers. A consideration of the calcion buffering Equation 1 shows that equivalent increments of CaCl_2 produce equivalent decrements in buffer salt, BHA. Therefore, the unit for the calcion buffer value is the differential ration $\frac{d[\text{BA}]}{dp\text{Ca}}$ which gives the relationship between the increment of calcium salt added to a calcion buffer solution and the resultant increment in $p\text{Ca}$. This unit for calcion buffer value in terms of logarithmic $p\text{Ca}$ is analogous to that introduced by Van Slyke (2) for the hydrion buffer value and has similar advantages. The buffer value is the number of gram equivalents of buffer salt necessary to change the calcion concentration one unit of $p\text{Ca}$.

The General Equation for Calcion Buffer Value.

The calcion buffer value may be determined by rewriting Equation 14,

$$\text{Ca}^{++} = K \frac{([\text{C}] - [\text{BA}])^n}{[\text{BA}]^{2n}}$$

where $[\text{C}]$ is the total acid concentration and $[\text{C}] - [\text{BA}]$ is the free buffer acid concentration. Expressing in logarithmic form,

$$p\text{Ca} = pK - n \log \frac{([\text{C}] - [\text{BA}])}{[\text{BA}]^2}$$

Differentiating with respect to $[\text{BA}]$ and inverting, we obtain for the calcion buffer value, ρ ,

$$\rho = \frac{d[\text{BA}]}{dp\text{Ca}} = -\frac{1}{n} \cdot \frac{d[\text{BA}]}{d \log \left(\frac{[\text{C}] - [\text{BA}]}{[\text{BA}]^2} \right)} = \frac{2.3}{n} \cdot \frac{[\text{BA}] ([\text{C}] - [\text{BA}])}{2 [\text{C}] - [\text{BA}]} \quad (16)$$

From the above and from the Henderson-Hasselbalch equation,

$$[BA] = \frac{K'a}{K'a + [H^+]} [C]$$

and

$$[HA] = \frac{[H^+]}{[H^+] + K'a} [C]$$

where $K'a$ is the acid dissociation constant divided by the fraction of the salt ionized. For polybasic acids, the concentration of the acid and basic intermediate compounds may be expressed by similar but more complex ratios. Within physiological conditions, however, the simpler relations suffice. For such systems, the $K'a$ value is the intermediate dissociation constant of the weak buffer acid divided by the fraction of the salt ionized.

Substituting these values in Equation 16 we obtain,

Equivalent expressions for the calcium buffer value, $\frac{d(BA)}{dpCa}$, or ρ .	$\left\{ \begin{array}{l} \\ \\ \\ \\ \end{array} \right.$	$\frac{d(BA)}{dpCa} = \frac{2.3}{n} \cdot \frac{[BA] [HA]}{[C] + [HA]} \quad (17)$
		$= \frac{2.3}{n} \cdot \frac{[BA] [HA]}{[BA] + 2[HA]} \quad (18)$
		$= \frac{2.3}{n} \cdot \frac{K'a [H^+] \cdot [C]}{(K'a + [H^+]) (K'a + 2[H^+])} \quad (19)$
		$= \frac{2.3}{n} \cdot \frac{K'a [HA] [C]}{(K'a + [H^+]) ([C] + [HA])} \quad (20)$
		$= \frac{2.3}{n} \cdot \frac{[H^+] [BA]}{(K'a + 2[H^+])} \quad (21)$

The accuracy of Equations 16 to 21 has been tested against experimental results by applying data reported in a previous paper (1). The values given in Table II show satisfactory agreement between the theoretically calculated calcium buffer values, i.e. $\frac{d[BHCO_3]}{dpCa}$ or ρ , and those approximated from the experimental data for $\Delta BHCO_3$ and ΔpCa .

Deductions from the General Equations.

These equations permit the following deductions:

- (1) The calcium buffer value at a given hydron concentration is directly proportional to the total molal acid concentration, $[C]$ (Equation 19).

(2) The calcion buffer value at a given hydron concentration is directly proportional to the buffer salt concentration, [BA] (Equation 21).

(3) The molal calcion buffer value, ρ_M , follows from Equation 19

$$\rho_M = \frac{d[BA]}{[C]dpCa} = \frac{2.3}{n} \cdot \frac{K'_a[H^+]}{(K'_a + [H^+])(K'_a + 2[H^+])} \quad (22)$$

The relationship between the absolute calcion buffer value, ρ , and the molal buffer value ρ_M , is

$$\frac{\rho}{[C]} = \rho_M$$

(4) The calcion buffer effect is independent of the nature of the buffer acid since Equations 16, 17, and 18 are independent of the dissociation constant of the acid. Phosphoric and carbonic acids have, in the presence of their salts, under similar conditions and equivalent concentrations corrected for valence differences, the same buffer effectiveness but their maximum calcion buffer action is exerted at different pH.

(5) *The Calcion Buffer Values of the Carbonates and Phosphates.*—These are given by Equations 16 to 21 in which the value of n is unity for the carbonates and two-thirds for the phosphates.

The Calcion Buffer Value of a Mixture of Calcion Buffers.—In a solution of a given hydron concentration $[H^+]$, containing several calcion buffers, the following relations hold at equilibrium,

$$[H^+][A^+]_i = K_i([C]_i - [A^+]_i)$$

where $i = 1, 2, 3, \dots, n$

The condition of electroneutrality requires that,

$$[B^+] + [H^+] = [OH^-] + [A^-]_1 + [A^-]_2 + \dots + [A^-]_n$$

where $[B^+]$ represents the total equivalent concentration of buffer salt cations present in the solution; in this summation both $[H^+]$ and $[OH^-]$ may be neglected, as they are small in comparison with the other quantities. Substituting for the anion concentrations their equivalents,

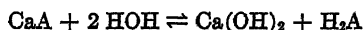
$$[BA] = \left(\frac{K'_1[C]_1}{K'_1 + [H^+]} + \frac{K'_2[C]_2}{K'_2 + [H^+]} + \dots + \frac{K'_n[C]_n}{K'_n + [H^+]} \right)$$

where the K' value is the intermediate dissociation constant divided by the fraction of salt ionized. Hence, the total buffer salt concentration is the sum of the separate buffer salts. Moreover, the differential coefficients of this sum of functions is equal to the sum of the differential coefficients of these functions, that is, the calcion buffer values, $\frac{d(BA)}{dpCa}$, are additive.

A solution containing several calcion buffers has for its resultant calcion buffer value at a given hydrion concentration, the sum of the individual buffer values,

$$\rho = (2.3) [H^+] \left\{ \frac{1}{n_1} \frac{[BA]_1}{(K'a_1 + 2[H^+])} + \frac{1}{n_2} \frac{[BA]_2}{(K'a_2 + 2[H^+])} + \dots \right\} \quad (23)$$

The Calcion Buffer Value of an Aqueous Highly Dissociated Calcium Salt.—Addition of a highly dissociated calcium salt to water yields a solution which exerts a certain amount of calcion buffer value. This may be determined by considering that addition of a calcium salt to water results in an hydrolysis, according to the equation,



Here a molecule of CaA is transformed to a molecule of the calcium buffer salt, $Ca(OH)_2$. Calcium hydroxide is a relatively insoluble compound whose solubility product is of an order of 10^{-16}

$$[Ca^{++}] [OH^-]^2 = k_1$$

or

$$Ca^{++} = \frac{k_1}{[OH^-]^2}$$

Inverting and expressing in logarithmic form,

$$pCa = pK_1 + 2 \log [OH^-]$$

Differentiating,

$$dpCa = 2d \log [OH^-]$$

Since a molecule of CaA is equivalent to a molecule of $Ca(OH)_2$, we may express the addition of a calcium salt by the formation of the calcium buffer salt, $Ca(OH)_2$, or symbolically by $2 BA$

But,

$$BA \rightleftharpoons \frac{1}{2} Ca(OH)_2 \rightleftharpoons \frac{2[OH^-]}{\gamma_b}$$

where γ_b represents the degree of dissociation of $Ca(OH)_2$.

Differentiating,

$$d[BA] = \frac{d[OH^-]}{\gamma_b}$$

Therefore,

$$\rho = \frac{d[BA]}{dpCa} = \frac{1}{\gamma_b} \frac{d[OH^-]}{dpCa} = \frac{1}{\gamma_b} \cdot \frac{d[OH^-]}{2d \log [OH^-]} = \frac{1}{2\gamma_b} \cdot \frac{[OH^-]}{0.4343} = \frac{1.15}{\gamma_b} [OH^-] \quad (24)$$

It can be similarly derived that,

$$\frac{d[BA]}{dpCa} = \frac{1.15}{\gamma_a} [H^+] \quad (25)$$

where γ_a is the degree of dissociation of the acid. Adding Equations 24 and 25, respectively, we obtain the total calcium buffer value of an aqueous, highly dissociated calcium salt solution.

$$\rho_w = \frac{d[BA]}{dpCa} = 1.15 \left(\frac{[H^+]}{\gamma_a} + \frac{[OH^-]}{\gamma_b} \right) \quad (26)$$

This means that any given $[H^+]$ or $[OH^-]$, highly dissociated calcium salt must be added at the rate of 1.15 gram equivalents per liter per unit change in pCa effected.

The total calcium buffer value of a weakly dissociated calcium salt solution to which highly dissociated calcium salts are added is the sum of the calcium buffer values of the weakly and the strongly dissociated calcium salts. This follows from Equation 23 and is given by the sum of Equations 22 and 25,

$$\rho = 1.15 \left(\frac{2K'_a[C] [H^+]}{(K'_a + [H^+]) (K'_a + 2[H^+])} + \frac{[H^+]}{\gamma_a} + \frac{[OH^-]}{\gamma_b} \right) \quad (27)$$

Since $[H^+]$ and $[OH^-]$ are usually negligible in comparison with the first member of Equation 26, the use of ρ_M becomes permissible within the physiological range.

The Maximum Calcium Buffer Values.

The maximum calcium buffer value is determined when $\frac{d\rho}{d[BA]}$ becomes zero. Repeating the differentiation of Equation 16 we obtain,

$$\frac{d\rho}{d[BA]} = \frac{1}{\log_{10} e} \left(1 - \frac{2[C]^2}{(2[C] - [BA])^2} \right) \quad (28)$$

When this expression becomes equal to zero

$$[BA] = (2 \mp \sqrt{2}) [C] \quad (29)$$

To determine which of these two values represents the maximum.

the necessary and sufficient condition is that $\frac{d^2\rho}{d[BA]^2} < 0$ for that value. Repeating the differentiation of Equation 28,

$$\frac{d^2\rho}{d[BA]^2} = \frac{-4[C]^2}{(2[C] - [BA])^3} \quad (30)$$

Substituting $(2 - \sqrt{2}) [C]$ from Equation 29 in Equation 30 a negative value is obtained; for $[C]$ is always positive. Hence the maximum calcion buffer value is determined when

$$[BA] = (2 - \sqrt{2}) [C] = 0.586 [C] \quad (31)$$

Hence the maximum calcion buffer effect is produced by any calcion buffering, weak acid-salt solution containing 0.586 part of buffer salt to 0.414 part of free buffer acid.

The Molal Calcion Buffer Value at the Maximum.— ρ_M at the point of maximum calcion buffering may be calculated from Equations 17, 18, and 19 by substituting for $[BA]$ its equivalent 0.586 $[C]$ from Equation 28. Hence,

$$\rho_M = \frac{2.3}{n} \cdot \frac{[BA] [HA]}{[C] + [HA]} = \frac{0.395}{n} \quad (32)$$

for any calcion buffering, weak acid-salt solution. Since the value of n for the carbonates is unity and for the phosphates is two-thirds, the molal calcion buffer value for the carbonates is 0.395 and for the phosphates is 0.592. At the maximum a molal solution of phosphates buffers the calcion concentration one and one-half times better than a molal solution of carbonates. The meaning of these values may be ascertained from the buffering reaction, from which it is evident that for every molecule of calcium salt added to the calcion buffering solution, a molecule of calcium buffer salt is formed which is transformed into a molecule of the insoluble normal calcium salt. Hence, at the isohydric point of maximum buffering, a highly dissociated calcium salt must be added or removed to the extent of 0.395 M to carbonates and 0.592 M to phosphates, to change the original calcium concentration to 10-fold or $\frac{1}{10}$ its own value, and thereby cause one unit change in pCa .

The Relation of pH to Maximum Calcion Buffer Value.—The pH of a buffer solution at which its molal calcion buffer value

is a maximum, may be determined directly from the Henderson-Hasselbalch equation,

$$\text{pH} = \text{pK}'_a + \log \frac{[\text{BA}]}{[\text{HA}]}$$

Since $[\text{BA}]$ and $[\text{HA}]$ are 0.586 and 0.414, respectively, at the maximum, the equation becomes,

$$\text{pH} = \text{pK}'_a + \log \sqrt{2} \quad (33)$$

and is indicated graphically in Fig. 2.

This equation may also be derived from the equivalent expressions given by Equations 19 to 21, by substituting the calculated ρ_M values at the maximum from Equation 32; *e.g.*,

$$\frac{2.3}{n} \cdot \frac{[\text{BA}] [\text{H}^+]}{(\text{K}'_a + 2[\text{H}^+])} = \frac{2.3}{n} \cdot \frac{(0.586) [\text{H}^+]}{(\text{K}'_a + 2[\text{H}^+])} = \frac{0.395}{n}$$

Solving for H^+ , we obtain,

$$\text{H}^+ = 0.7 \text{K}'_a$$

Inverting and expressing in logarithmic form,

$$\text{pH} = \text{pK}'_a + \log \sqrt{2}$$

which is identical with Equation 33.

The independent derivation of this equation follows from direct differentiation of ρ given by Equation 22,

$$\frac{d\rho_M}{d\text{pCa}} = \left(\frac{d\rho_M}{d[\text{H}^+]} \right) \left(\frac{d[\text{H}^+]}{d\text{pH}} \right) \left(\frac{d\text{pH}}{d\text{pCa}} \right) \quad (34)$$

Differentiating ρ_M with respect to H ,

$$\frac{d\rho_M}{d[\text{H}^+]} = \frac{2.3 (\text{K}'_a^3 - 2 \text{K}'_a [\text{H}^+])^2}{(\text{K}'_a + [\text{H}^+]) (\text{K}'_a + 2[\text{H}^+])^2}$$

Since,

$$\frac{d[\text{H}^+]}{d\text{pH}} = -2.3 [\text{H}^+]$$

and from the relations,

$$\text{Ca}^{++} = \frac{k}{[\text{OH}^-]^2} = \frac{k_1}{k_2^2} [\text{H}^+]^2 = k_2 [\text{H}^+]^2$$

or,

$$\text{pCa} = \text{pK}_2 - 2 \log [\text{H}^+]$$

and on differentiating,

$$\frac{d[\text{H}^+]}{d\text{pCa}} = -\frac{1}{2}$$

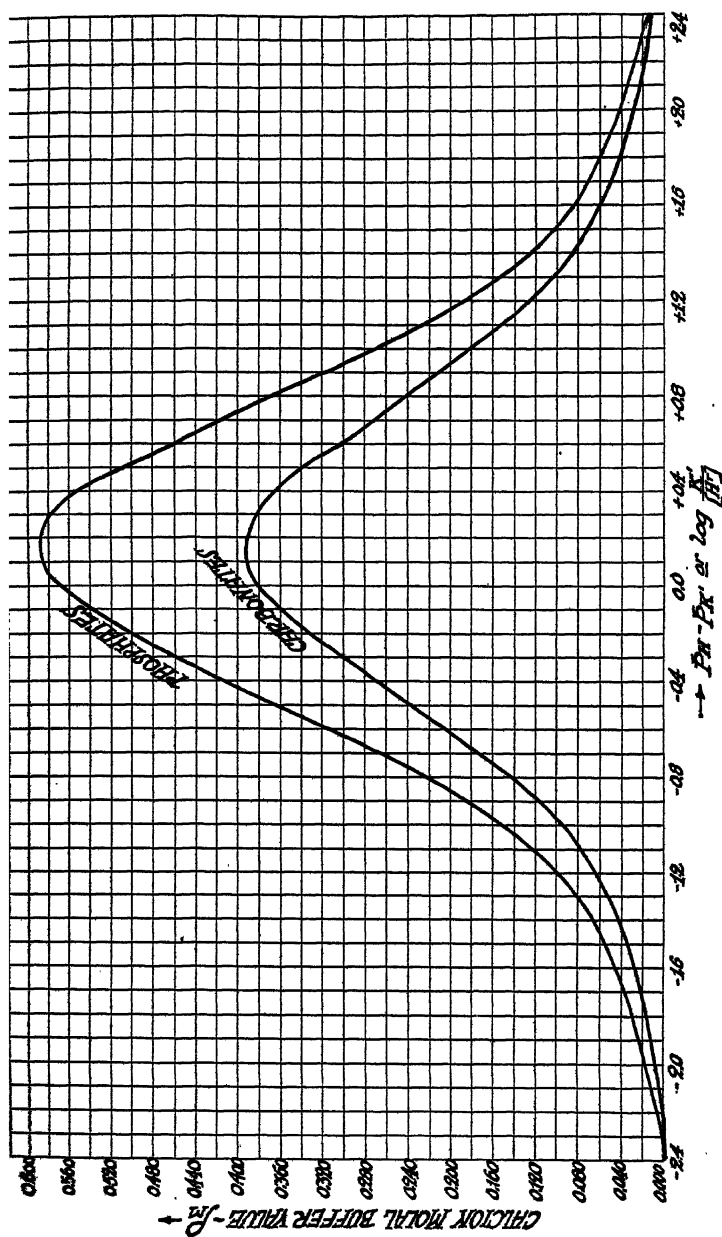


Fig. 2. Curves for the calcium molar buffer values of the carbonates and phosphates, calculated from the equation,

$$p_M = \frac{2.3}{n} \frac{K'_a [H^+]}{(K'_a + [H^+]) (K'_a + 2[H^+])}$$

Equation 34 becomes on multiplying these three differentials,

$$\frac{d\rho_m}{dpCa} = \frac{1.15[H^+](K'a^2 - 2K'a[H^+])^2}{(K'a + [H^+])(K'a + 2[H^+])^2}$$

Making this equation equal to zero,

$$H^+ = \pm \frac{K'a}{\sqrt{2}}$$

Proceeding as above to determine which of these values is a maximum, inverting and expressing in logarithmic form, we obtain,

$$pH = pK'a + \log \sqrt{2}$$

The ratio of buffer salt to free buffer acid is the same for any calcium buffering solution at the maximum and hence the pH at that point for any buffer concentration may be calculated from Equation 33 by using $pK'a$ values of 6.15 for the carbonates and 6.85 for the phosphates. *The maximum calcium buffer value for the carbonates is at pH 6.30 and that for the phosphates is at pH 7.00.*

The Molal Calcium Buffer Value at the pH of the Blood.—The carbonates and phosphates exert their maximum calcium buffer effect at an hydron concentration greater than that of normal blood. The calcium buffer value at pH 7.35 may be read directly from the plotted curves, Fig. 2, or may be calculated from Equation 19. At pH 7.35 the molal calcium buffer value of the carbonates is 0.111 or 28 per cent of the maximum. The molal calcium buffer value of the phosphates is 0.265 or 45 per cent of the maximum.

The Calcium Buffer Value of Blood Serum.

The calcium buffer value of blood may be calculated from Equations 17 to 22. Since the corpuscles contain no calcium, calcium buffering is limited to the serum. Normal blood serum has a total bicarbonate concentration of about 0.03 N and an hydron concentration of 0.45×10^{-7} . Hence, the calcium buffer value of the serum carbonates is 3.5×10^{-3} from

$$\rho = \frac{d[BA]}{dpCa} = \frac{2.3}{n} \frac{K'a[H^+] \cdot [C]}{(K'a + [H^+])(K'a + 2[H^+])}$$

At the same hydron concentration and at a total phosphate concentration of 0.001 M the calcium buffer value of the serum phosphates is 0.5×10^{-3} . The combined calcium buffer value of the

carbonates and phosphates of normal blood serum according to Equation 23 is 4.0×10^{-3} . Therefore, at physiological conditions, the calcion buffer value of the carbonates is seven times that of the phosphates.

SUMMARY.

1. The calcion concentration is regulated by calcion buffers. They are electrolytes which resist the change in calcion concentration upon addition of calcium salts. Calcion buffers are mixtures of weak acids and their salts which react to form insoluble, normal calcium salts and soluble, intermediate calcium salts.

2. The calcion concentration of any calcion buffering solution is determined by the ratio of the concentrations of the free buffer acid, HA, to the buffer salt, BA, according to the relation,

$$\text{Ca}^{++} = K \frac{[\text{HA}]^n}{[\text{BA}]^{2n}}$$

where n is the ratio of the valence of calcium to that of the acid, and K is an equilibrium constant.

3. Calcion concentrations may be expressed in logarithmic units as $\log \frac{1}{[\text{Ca}^{++}]} = \text{pCa}$ which is given by the general equation,

$$\text{pCa} = \text{pK} + n \log \frac{[\text{BA}]^2}{[\text{HA}]}$$

4. The calcion pK is 4.2 at 38° for the carbonates as calcion buffers.

5. The unit for the calcion buffer value of a solution is the number of gram equivalents of calcium salt or acid necessary to change the calcion concentration one unit of pCa . This is expressed by the differential ratio $\frac{d[\text{BA}]}{d\text{pCa}}$ which defines the calcion buffer value at any given calcion concentration.

6. The general equation for the calcion buffer value ρ , is

$$\rho = \frac{d[\text{BA}]}{d\text{pCa}} = \frac{2.3}{n} \cdot \frac{K'a[\text{C}] \cdot [\text{H}^+]}{(K'a + [\text{H}^+]) (K'a + 2[\text{H}^+])}$$

This equation defines the calcion buffer value of the carbonates for which n is unity and that of the phosphates for which n is two-thirds.

7. The calcion buffer value, at any given hydron concentration, is directly proportional to the total concentration of buffer acid or salt.

8. The calcion buffer value is independent of the nature of the weak acid provided it forms an insoluble, normal calcium salt.

9. The calcion buffer value of a mixture of calcion buffers is the sum of the separate calcion buffer values.

10. The maximum calcion buffer value is attained when there are 0.586 part of buffer salt and 0.414 part of free buffer acid.

11. The molal calcion buffer value at the maximum is given by the ratio $\frac{0.395}{n}$.

12. The pH at which the calcion buffer value is a maximum is given by,

$$\text{pH} = \text{pK}'a + \log \sqrt{2}$$

which is pH 6.30 for the carbonates and pH 7.00 for the phosphates.

13. The molal calcion buffer value at pH 7.35 is 0.111 or 28 per cent of the maximum for the carbonates and 0.265 or 45 per cent of the maximum for the phosphates.

14. The calcion buffer value of the carbonates of normal blood serum at pH 7.35 is 3.5×10^{-3} and that of the serum phosphates is 0.5×10^{-3} . The combined calcion buffer value of blood serum is 4.0×10^{-3} .

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A NOTE ON THE RELATION OF pH TO TUNGSTIC ACID PRECIPITATION OF PROTEIN.*

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In the precipitation of nitrogenous material by the tungstic acid method of Folin and Wu (1) a certain degree of acidification is necessary. Rumsey (2) placed this on a pH basis and found that maximum precipitation of nitrogen from cereal extracts and best clarification is obtained in the range pH 2 to 1.3. Hanzlik (3) showed that dialyzed horse serum is best precipitated with phosphotungstic on the acid side of the isoelectric point of serum protein.

In an examination of diphtheria antitoxin serum the following interesting data were obtained. At a pH of about 5.0, which is on the acid side of the isoelectric point of serum globulin, the zone of maximum precipitation of nitrogen occurs. A value of 0.10 per cent protein-free nitrogen is obtained in the filtrate. At pH 2.8 the protein-free nitrogen decreased slightly to 0.08 per cent and this constant was found at all pH values determined below this and at the acidity of 10 per cent acid solution (see Table I). Folin's proportions of acid to tungstate gave a pH of 2.8 which is within the range of maximum nitrogen precipitation.

The determination of protein-free nitrogen in the serum by the trichloroacetic acid method of Greenwald (4) gave identical results with those obtained by tungstic acid at the pH of maximum precipitation. The pH of the trichloroacetic acid filtrate was less than pH 1.2. The agreement of protein-free nitrogen determinations by the two methods indicates absence of peptone in the serum according to Hiller and Van Slyke (5).

*Approved for publication by the Surgeon General.

258 pH to H_2WO_4 Precipitation of Protein

No material change in total or non-protein nitrogen as a result of autolysis was found in serum kept at room temperature for a period of 3 months.

TABLE I.
Diphtheria Antitoxin Serum.

4.0 gm. serum + 0.5 gm. $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ per 100 cc.

2/3 N H_2SO_4 acid.	pH	Filtrate nitrogen.
cc.		per cent
2.8	5.9	2.45
3.0	5.8	2.24
3.2	5.6	1.41
3.4	5.4	0.86
3.5	5.3	0.45
3.6	5.3	0.22
3.8	5.0	0.10
4.0	4.6	0.09
5.0	2.8	0.08
6.0	2.5	0.07
7.0	2.1	0.08
8.0	1.9	0.07
10.0	1.7	0.08
30.0	1.0	0.08
50.0*		0.07

*20 per cent sulfuric acid.

TABLE II.
Peptone.

0.8 gm. peptone + 2.0 gm. $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ per 100 cc.

2/3 N H_2SO_4 acid.	pH	Filtrate nitrogen.
cc.		per cent
14.0	4.3	13.42
18.0	2.6	8.07
21.0	2.0	6.98
25.0	1.7	6.76
50.0	1.0	5.81
25.0*		4.72
50.0*		4.99

*20 per cent sulfuric acid.

In contrast to these results, obtained by the precipitation of nitrogen in serum by tungstic acid, are the results obtained in peptone solutions. The nitrogen precipitated from peptone solu-

tions by tungstic acid increased considerably as the pH decreased from pH 4.0 to 1.0, with a very slight reversal of precipitation at an acidity of 10 per cent acid solution, as shown in Table II.

The peptone nitrogen is only slightly precipitated at a pH of 4.0 while at this acidity the protein in the serum is completely precipitated. There is no known pH at which peptide nitrogen is precipitated separate from other nitrogen compounds in peptone solution.

The quantity of nitrogen precipitated is also affected by a change in the proportion of tungstic acid to peptone, or the concentration of reagents in solution.

It is known that freshly prepared tungstic oxide is soluble in aliphatic amines (6), in salts of tartaric (7), citric, and malic acids (8), and in oxalic acid (9). Folin and Wu (1) warn against excess of citrate or oxalate in blood used for protein precipitation with tungstic acid.

It is now suggested that the tungstic acid precipitate in peptone solutions is affected by the various peptones, bases, amino acids, and other organic acids probably present in the peptone.

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FAT EXCRETION.

II. THE QUANTITATIVE RELATIONS OF THE FECAL LIPOIDS.

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In an earlier paper (1) it was shown that there was always fatty material in the feces whether fat was present in the food or not; that addition of moderate amounts of fat to the diet did not increase the feces fat to any considerable extent; and that the composition and properties of the excreted fatty material were largely independent of the fat of the food. The comparative constancy in amount and composition of the fecal fat under a variety of conditions indicated that it was not to be regarded as unabsorbed food fat but rather of the nature of an excretion, whether directly from the blood or indirectly through the normal intestinal secretions. The possibility of its origin in desquamated epithelial cells or in intestinal bacteria could not, of course, be excluded. Recent work by Holmes and Kerr (2) has led them to similar conclusions as to the constancy of composition of the fecal fat and its independence of the fat of the food. The present work was undertaken in order to obtain quantitative information regarding the different substances making up the fecal lipid and their relations to each other and to the lipid of the food, with the purpose of discovering the source of the feces fat and its bearing on fat metabolism.

EXPERIMENTAL.

The general procedure consisted of feeding experimental animals various standard diets with and without fats of different composition over periods of a week during which time the feces were

collected, and the fat was extracted and analyzed. The details of the work are as follows:

Animals and the Technique of Handling.—Largely because they required less of the sometimes difficultly prepared food, cats were first used as experimental subjects. Between the experimental periods there was always a rest period of at least 2 weeks during which they were fed all they would eat of a diet of bread, liver, and dog biscuit. When the experimental diet was first given it was always found difficult to make the cats eat, a period of 4 to 10 days being required before they were eating it completely in all cases, so that the preexperimental conditions consisted of a period of partial fasting and partial eating of the diet in question for approximately a week before the actual experiment was started. The experiments were each of 1 week's duration, with the periods marked off by charcoal. The feces were collected daily in covered beakers. The animals were given some exercise each day while their cages were being cleaned and food was being prepared. It was found in some preliminary experiments, using a normal diet of bread and liver, that a diet computed on the basis of 90 calories per kilo of body weight gave the optimum results and the diets used were computed on this basis. The animals were weighed every 2nd day and remained in good condition throughout all the experiments.

Because of the difficulty of getting them to eat and the tendency of the feces to be soft even when large amounts of kaolin or bone ash were fed, cats were finally abandoned as subjects in favor of dogs. The technique of handling dogs was the same as for cats except that Cowgill's (3) method of calculating the diet was used. It was not found necessary to feed vitamins or a salt mixture during the weekly period. In the case of the coconut oil and fat-free diets a better mixture was obtained and the animals ate more readily if a little meat extract, dissolved in water, was mixed with the food. The dogs ate the diets completely and remained in good condition during the experiments, while the feces were hard and easy to handle in all cases.

Food Materials. Carbohydrate-Starch.—The starch used in the fat-free diet (cats) was Kingsford's corn-starch dextrinized by spreading out to a depth of $\frac{1}{4}$ to $\frac{1}{2}$ inch on a large shallow tray and heating carefully in a hot oven until just browned. After using

this material in the fat-free diet of the cats an article by Taylor and Nelson (4) came to our notice, calling attention to the fact that practically all starch contained fat. Analysis of the dextrinized material for fat by their method showed a content of about 0.9 per cent. This amount was probably not sufficient to affect the results materially, but partly on this account and partly because dextrin was easier to use it was deemed best to abandon starch as a basal carbohydrate in the fat-free diets and to substitute commercial dextrin for it. However, the dextrin was also found to contain fat when analyzed by the method of Taylor and Nelson, a 25 gm. sample yielding 0.086 gm. of fat. Extraction with hot alcohol in the continuous extractor described by Clarke, Adams, Conant, and Kamm (5), lowered the fat content as follows, analysis of 25 gm. samples being made at the end of 2 hour periods.

Original content.....	gm.
After 2 hrs. extraction.....	0.086
“ 4 “ “	0.028
“ 6 “ “	0.023
“ 8 “ “	0.009
“ 8 “ “	0.005

All the dextrin used was therefore extracted for 8 hours.

Casein.—Ordinary commercial casein was used after a preliminary 16 hour extraction with alcohol in the apparatus described above. 16 hours extraction reduced the fat content to 0.04 per cent which was considered negligible for these experiments.

Coconut Oil.—The coconut oil was fed in the form of the commercial margarine preparation “Nucoa” because it contained less of the objectionable odor than the commercial oil. The iodine number of this material was found to be 10.

Olive Oil.—The iodine number of the olive oil was 96.4.

Meat Extract.—Liebig’s meat extract was used.

The make-up of the various diets is as follows:

Normal diet (cats) per kilo per day.	Liver	gm. 26.5
	Dog biscuit.....	19.8
	Kaolin.....	66.0
Fat-free diet (cats) per kilo per day.	Starch.....	17.0
	Casein.....	5.82
	Bone ash.....	1.12
	Meat extract.....	1.50

		gm.
Fasting diet—sham-feeding (cats) per cat per day.	Agar-agar.....	1.0
	Bone ash.....	5.0
	Meat extract.....	4.0
Coconut oil diet (cats) per kilo per day.	Casein.....	7.0
	Dextrin.....	12.1
	Coconut oil.....	1.5
	Kaolin.....	5.0
Olive oil diet (dogs) per kilo per day.	Casein.....	6.4
	Olive oil.....	3.5
	Sugar.....	5.7
	Bone ash.....	0.4
Fat-free diet (dogs) per kilo per day.	Casein.....	6.4
	Sugar.....	13.6
	Bone ash.....	0.4
	Meat extract.....	0.35
Fasting diet—sham-feeding (dogs) per dog per day.	Agar-agar.....	1.8
	Bone ash.....	2.5
	Meat extract.....	2.5
Coconut oil diet (dogs)* per kilo per day.	Casein.....	6.4
	Coconut oil.....	3.5
	Sugar.....	5.7
	Bone ash.....	0.4
	Meat extract.....	0.35

The sham-feeding diet was made up of agar jelly and bone ash and flavored with meat extract. Since the only nutritive material in this diet was the small amount of meat extract (4 gm. per day for the cats, 2.5 gm. per day for the dogs) the animals may be regarded as fasting. The advantage of this sham-feeding over actual fasting is that the normal movements and possibly also the normal secretions of the intestine are kept up.

Analytical Methods.

The fat in the feces was analyzed largely by the method used by Bloor (6) for blood plasma with certain modifications. The method as used in the normal diet experiment was as follows: As soon as the collections were complete the feces were covered

* Dogs 23-7 and 23-2 were given the diet as shown. For Dogs 24-7 and 24-8 the diet was increased to 1.13. For Dogs 24-10 and 24-11 the diet was increased to 1.06.

with 20 per cent NaOH (350 to 600 cc.) and heated on the steam bath for 24 hours, after which the mixture was carefully neutralized with concentrated HCl and extracted with ether until the ether was colorless. The ether extract was washed once with water to remove the HCl, the ether distilled off, and the residue evaporated to dryness by heating on the steam bath from 2 to 4 hours. When cool 100 cc. of petroleum ether (fractol) were added and, after solution had taken place, the mixture was allowed to stand overnight. In the morning the petroleum ether solution was filtered into a weighed flask, and the tarry residue washed twice with fresh solvent. The combined solvent was distilled to small volume, the residue dried 1 hour on the steam bath, and the flask weighed to give the total lipoids.

Separation of Unsaponifiable and Fatty Acid Fractions.—The total lipid was dissolved in 100 cc. of fractol, used in several portions, and transferred to a separating funnel to which were added 100 cc. of 0.1 N alcoholic KOH and 100 cc. of distilled water. The whole was shaken and tested with litmus paper. If not alkaline a few cubic centimeters of concentrated alcoholic KOH were added. When the two layers had separated the lower was drawn off, the upper filtered into a weighed flask in which it was concentrated by distillation while the lower layer was poured back into the funnel and shaken up with a new portion of fractol which in turn was separated and added to the concentrate in the weighed flask and again concentrated. This process was repeated once more after which the flask was heated an hour on the steam bath and weighed to give the unsaponifiable fraction.

The lower layer was acidified with HCl and extracted with fractol to give the fatty acid fraction which was dried and weighed as before. A melting point tube was filled with this material by placing an open end of the tube in the melted acids, allowing some to rise into the tube by capillary attraction and sealing the tube. The melting and solidification points were taken the next day.

Volatile Fatty Acids.—In the earlier part of the work the volatile fatty acid was determined as follows: The fatty acid fraction was dissolved in a little ether and transferred to a 600 cc. flask, containing 400 cc. of water and 50 cc. of H_2SO_4 (1:4 by volume) and equipped with a Hopkins distilling bulb which was connected to a condenser. The solution was distilled into a flask

containing standard (approximately 0.1 N) NaOH until it no longer came over acid to litmus. Phenolphthalein was added to the receiver and as the color disappeared more standard alkali was added. The excess was titrated with HCl. The volume in the distilling flask was not allowed to become less than 150 cc., more water being added as needed. After volatile acids had been determined as above for normal, fat-free, and fasting experiments with cats, the data given by Lewkowitsch and Warburton (7), regarding the extent of evaporation of volatile acids, were noticed and it was realized that inasmuch as one lot of material containing volatile acid had been heated on the steam bath for four periods of at least an hour each, undoubtedly a large percentage and probably nearly all the volatile acid had been lost. Also, it was found that when the liquors from the separation of the unsaponifiable and fatty acid fractions from the sham-feeding diet (acid with sulfuric acid) were distilled an amount of volatile acid as great as, or greater than, that recovered by the regular distillation was obtained, showing that an appreciable amount had been lost in the 50 per cent alcoholic solution at this point. It was necessary, therefore, so to modify the procedure as to prevent this loss of volatile acid and such a modification was attempted in working up the feces from the coconut oil diet. The feces were digested as before, but neutralized with 10 N H_2SO_4 instead of HCl. It was found impossible to extract the fat from the first two samples neutralized, due to the formation of a stable emulsion. Centrifuging did not break the emulsion and filtration was tried, using suction. This was a slow process requiring $1\frac{1}{2}$ days and probably involved some loss of volatile acid, but it was possible to extract both the precipitate and the filtrate with little difficulty. This suggested the idea of filtering the material hot. The neutralization heats the solution almost to boiling and while still hot it was poured on a suction filter and found to filter rapidly, the precipitate being sucked dry in less than 15 minutes. Without washing, the precipitate was transferred back to the digestion beaker, the funnel was thoroughly washed with ether into the beaker, the material stirred well, covered, and allowed to stand 15 minutes when it was filtered and washed with ether until the filtrate came through colorless. While this ether extract was being concentrated the first filtrate obtained above was extracted

with ether and the ether added to the concentrate from the extraction of the precipitate. As before this extraction was continued until the ether came colorless. After the last extraction the solution was concentrated to about 50 cc. and transferred to the volatile acid distillation apparatus described above and distilled in the same way except that a stronger (5 N) standard alkali was used, due to the greatly increased amount of volatile acid. The residue from distillation was extracted with fractol to give total lipid minus volatile acid and the procedure from this point on for the separation of the various lipid fractions was the same as above, with the omission, of course, of the distillation of volatile acids.

It was essential to obtain some idea of the composition of the volatile acids in order that they might properly be reckoned in with the total lipid. The titrated volatile acid distillates from the fat-free and coconut oil diets (dogs) were united, made alkaline, concentrated to about 200 cc., acidified with H_2SO_4 , and extracted with fractol. The fractol solution was distilled in a small distilling flask. About half the residue after the solvent had come off distilled between 150 and 175°, leaving non-volatile material behind which solidified on cooling. There is always in volatile acid distillations considerable white, flaky, insoluble material in the distillate and it was thought that this undistilled residue consisted of this neutral material. Some experiments show, however, that there is probably some fatty acid in this residue. This would probably not appreciably affect our results; but in the continuation of the work as we are now carrying it on more attention is being paid to the volatile fraction. The distillates were determined by the method of Dyer (8). The results obtained showed this material to be mainly butyric acid and it has been so computed in our tables.

Separation of Solid and Liquid Fatty Acids.—The residue from the first volatile acid distillation was transferred to a separatory funnel and extracted with fractol. The unsaponifiable fraction was removed and the mixture of solid and liquid fatty acids was separated by the method of Twitchell (9) with a slight modification as follows: A sample of 2 gm., or the whole residue if less than that amount, was dissolved in alcohol (25 cc. per gm.), brought to a boil, an equal volume of boiling 3 per cent alcoholic lead acetate

solution added, and the whole boiled 2 minutes and allowed to stand overnight.

Next day the flocculent precipitate was filtered, the filtrate tested for lead with alcoholic H_2SO_4 , and the precipitate washed five or six times with small portions of alcohol. The precipitate was transferred quantitatively to the precipitation flask, a volume of alcohol equal to the total volume first used was added together with 0.5 cc. of glacial acetic acid, and the mixture brought to a boil and filtered from the tarry residue, which was washed twice with small portions of boiling alcohol. The filtrate was allowed to stand overnight and the precipitate which formed in it was filtered and washed three or four times with alcohol. The filtrate and washings were added to the filtrate from the first precipitation.

The precipitate was transferred back to the precipitation flask with 50 cc. of ether and to this mixture were added 20 cc. of water and 0.5 cc. of nitric acid (sp. gr. 1.2). The flask was shaken gently to bring all the lead soaps into contact with the acid, the whole transferred to a separating funnel, the acid layer separated, and the ether solution washed with successive portions of water until it was alkaline to methyl orange. This usually required six or seven washings. The washed ether solution was distilled and the residue dried to give the solid fatty acid fraction. A melting point was taken of this fraction.

Liquid Acids.—The combined alcoholic filtrates were distilled to about 10 cc. and 50 cc. of water containing 1 cc. of concentrated HCl were added. The lead chloride usually flocculated fairly well and caused no trouble, but sometimes it formed a fine suspension in the fractol used for extraction and it was necessary to centrifuge to clear it. Otherwise the solution was extracted by fractol as usual to give the liquid fatty acid fraction.

The iodine number of this fraction was determined, using the Hanus method.

A great deal of difficulty was experienced in working up the feces from the fat-free diet, due to the fact that after saponification and neutralization the whole mass formed a thick paste strongly suggestive of starch paste. It was considered probable that this was actually due to unabsorbed starch in the feces. The only method of extraction that would work at all was the laborious one of stirring the mass as thoroughly as possible with ether and centrifuging to separate, and this process was used.

Probably the extraction was not complete in this series, due to the difficulties mentioned. Otherwise the method was as given above.

The same method was used throughout in the sham-feeding experiment except that in the separation of neutral from fatty acid fractions, H_2SO_4 was used to acidify instead of HCl .

Fractionation of Liquid Acids.—In certain experiments the liquid acids in excess of the material required for the iodine number determinations were united and separated by the barium soap-benzene method as given by Brown and Beal (10) with modifications made necessary by the difference in amount. It was hoped by this process to separate the solid acids (carried through from the lead soap-alcohol separation) and the oleic from any more unsaturated acids which might be present. The modified method was as follows:

The combined liquids were freed from fractol by short heating on the steam bath, weighed, and neutralized by half normal alkali. The acids were usually quite dark in color making the end-point difficult to see and usually a small excess of alkali was added. To this solution was added a 6 per cent solution of BaCl_2 , slowly with stirring, until a sudden coagulation of the precipitate showed that an excess had been added. The precipitate was filtered, washed, transferred back to the precipitation flask, and heated $\frac{1}{2}$ hour on the steam bath. The precipitate agglutinated and squeezed out some solution which was decanted. The agglutinated material was washed twice by decantation and then dried by an air jet. It was next treated with benzene containing 5 per cent alcohol at the rate of about 7 cc. for each gram of acid being separated and refluxed until the material was almost or all dissolved. The solution was decanted and the flask washed by boiling up with small portions of benzene twice more. The decantate was placed in the ice box overnight.

Next day the gelatinous precipitate was filtered and washed with benzene until the filtrate was colorless. The almost white precipitate was decomposed by HCl (1:3) and extracted with fractol. The fractol solution was washed with water until barium-free, the fractol distilled, the residue dried, weighed, and the iodine number determined.

The filtrate was also treated with HCl , boiled to remove the benzene, and after cooling extracted with fractol. This fractol

solution was washed to remove barium, the fractol distilled, the residue dried, weighed, and the bromo derivatives were made and separated by the method of Baughman and Jamieson (11) with the following change. The ether solution was washed with sodium thiosulfate, then water, the ether distilled, and the residue dried on the water bath and weighed. The ether-insoluble portion was a white solid, while the ether-soluble portion was a very thick dark syrup.

In certain experiments bromine instead of iodine was used for the determination of the halogen absorption number with the intention of separating the resultant bromo acids. In the case of the coconut oil diet the combined chloroform residues were washed with water, the chloroform was distilled, and the residue dried on the steam bath. A tarry, unworkable mass, mostly insoluble in ether, was obtained and discarded. The chloroform residues of bromo acids from the fat-free, fasting, and olive oil diets (dogs) were washed with 5 per cent sodium thiosulfate solution, water, and distilled in vacuum. The temperature remained below 20°C. throughout the distillation except near the end when it rose to about 30°C. The vacuum was about 80 mm. The residue was a thick syrup, fairly light in color, and was washed into a weighed centrifuge tube by dry ether (10 cc. per gram of liquid acids used for iodine number determinations) allowed to stand in the ice box overnight, and treated as in the procedure used above. The ether-insoluble portion was an almost black, amorphous precipitate, while the ether-soluble portion was a thick dark syrup of the same appearance as that obtained by the other method.

Bromine Determinations.—These bromine derivatives were determined by the Parr bomb method as described by Brown and Beal (10). The solutions were standardized by NaCl and NaBr which had been dried at 110°C. for 12 hours and the method was checked by an analysis of pure *p*-bromoacetanilide and *p*-bromoaniline.

	Theory.	Obtained.
	<i>per cent</i>	<i>per cent</i>
<i>p</i> -Bromoaniline.....	46.51	46.44
<i>p</i> -Bromoacetanilide.....	37.38	36.58
		37.46

TABLE I.
Normal Diet (Cats).

Cat No.	Weight.	Total lipoid.	Unsataponifiable.			Non-volatile fatty acids.			Volatile fatty acids.		Total fatty acids.		Solid fatty acids.			Liquid fatty acids.		Iodine No.			
			Weight.		Total lipoid. (100 B) (A)	Total lipoid. (100 D) (A)	Melt- ing point.	Weight as but- tyric acid. E	Total fatty acids. (100 E) (F)	Weight. (D+E) (100-G)	Non- volatile fatty acids. (100 G) (D)	Weight.	Melt- ing point.	Non- volatile fatty acids. (100 H) (D)	Weight.						
			A	B	C											D	per cent		per cent	per cent	per cent
1	2.72	3.684	0.606	19.4	1.99	54.4	46	0.0141	1.40	1.004	80.6	0.363	18.12	55.5	1.28	64.0	48				
2	3.29	7.979	1.280	16.0	4.04	63.0	42	0.384	7.65	5.02	84	1.788	38.4	55	2.56	55.4	95.8				
4	3.88	5.570	0.920	16.5	2.99	56.6	43	0.171	5.41	3.16	83.5	0.958	32.1	57	1.75	58.6	81.6				
5	2.75	4.632	0.894	19.3	3.03	66.7	42.5	0.0506	1.61	3.08	80.7	1.178	39.5	54	1.532	50.6	92.4				
6	3.66	1.429	0.155	10.88	0.60	44.0	38.5	0.0268	4.28	0.626	87.2	0.1006	16.8	55	0.441	73.5	76				
8	3.63	1.151	0.168	14.60	0.564	52.2	40	0.0383	6.35	0.602	85.4	0.100	17.9	55	0.385	68.2	88				
9	2.66	1.984	0.679	34.20	0.440	26.8	28	0.0926	17.4	0.532	65.8	0.0777	17.7	52.5	0.359	81.5	76.6				
verage....	3.22	3.775	0.684	18.70	2.03	51.9	40	0.111	6.30	2.00	81.0	0.652	25.9	54.7	1.187	64.6	79.8				

TABLE II.
Fat-Free Diet (Cats).

Cat No.	Weight.	Total lipoid.	Unsatifiable.			Non-volatile fatty acids.			Volatile fatty acids.		Total fatty acids.		Solid fatty acids.		Liquid fatty acids.		Iodine No.
			Weight.	Total lipoid. (100 B). (A)	Weight.	Total lipoid. (100 D). (A)	Melt- ing point.	Weight as butyric acid. (100 E).	Total fatty acids. (100 E).	Weight. (D+E).	Total lipoid. (100-C).	Weight.	Non-volatile fatty acids. (100 G).	Melt- ing point.	Weight.	Non-volatile fatty acids. (100 H).	
		A	B	C	D	(A)		E	(E)	F		G	(D)		H	(D)	
	ka.	gm.	gm.	per cent	gm.	per cent		per cent	per cent	gm.	per cent	gm.	per cent		gm.	per cent	
2	3.58	3.518	0.212	6.04	1.30	45.4		0.295	18.5	1.59	93.96	0.426	32.23	51	1.155	88.84	74.6
3	3.82	5.757	0.389	6.75	2.61	55.4		0.578	18.2	3.19	93.25	0.830	31.80	47	2.22	85.44	62.0
4	3.54	1.650	0.161	9.75	1.00	62.2		0.132	11.7	1.13	90.25	0.140	14.00	48	0.852	85.2	69.7
5	2.20	3.600	0.337	9.36	2.00	60.9		0.196	8.9	2.20	90.64	0.596	29.81	49	1.402	70.1	70.6
8	3.42	3.310	0.375	11.32	1.91	62.1		0.149	7.2	2.05	88.68	0.584	30.62	50	1.240	64.93	78.5
9	2.45	2.561	0.195	7.60	1.53	63.6		0.169	10.00	1.69	92.40	0.301	19.67	55	1.310	85.62	75.4
range....	3.17	3.399	0.278	8.47	1.73	58.3		0.253	12.41	1.96	91.53	0.479	26.35	50	1.365	80.02	71.8

TABLE III.
Fasting Cats (Sham-Feeding).

No.	Weight.	Total lipid.	Unsataponifiable.			Non-volatile fatty acids.			Volatile fatty acids.		Total fatty acids.		Solid fatty acids.			Liquid fatty acids.		Iodine No.
			Weight.	Total lipid. (100 B) (A)	C	D	Total lipid. (100 D) (A)	Melt- ing point.	Weight as butyric acid. (100 E)	Total fatty acids. (100 F)	Weight. (D+E)	Total lipid. (100—C)	Weight.	Non- volatile fatty acids. (100 G)	Melt- ing point.	Weight.	Non- volatile fatty acids. (100 H)	
2	4.38 (3.54)*	1.89	0.756	40.00	1.02	53.95	43	0.026	2.47	1.05	60.0	0.295	28.8	54	0.672	65.8	80.0	
3	4.82 (3.68)	2.52	0.985	39.3	1.30	51.58	36	0.016	1.21	1.32	60.7	0.411	31.6	51	0.810	64.0	66.0	
4	3.60 (3.22)	1.81	0.718	39.6	0.931	51.39	39	0.021	2.36	0.952	60.4	0.291	31.2	52	0.606	65.0	66.6	
5	2.61 (2.04)	0.86	0.307	35.8	0.486	50.69	40	0.031	6.52	0.517	64.20	0.155	31.9	53	0.344	70.5	72.6	
3	4.00 (3.28)	2.69	1.017	37.8	1.38	51.30	39	0.037	2.69	1.41	72.2	0.432	31.3	54	0.968	70.0	61.4	
3	4.18 (3.34)	2.60	1.167	44.7	1.25	48.03	41	0.047	3.65	1.29	55.3	0.321	25.6	55	0.818	65.5	51.2	
2	3.06 (2.41)	1.92	0.65	33.75	1.12	58.03	40	0.022	1.97	1.14	66.25	0.392	35.0	48	0.675	60.2	78.0	
range.	3.81 (3.07)	2.04	0.80	38.71	1.07	50.25	39.7	0.0286	2.67	1.10	62.71	0.328	30.6	52.4	0.699	65.1	68.8	

Weights of animals at end of fasting period.

TABLE IV.
Coconut Oil Diet (Cats).

t No.	Weight.	Total lipoid.	Unsataponifiable.			Non-volatile fatty acids.			Volatile fatty acids.			Total fatty acids.		Solid fatty acids.				Liquid fatty acids.		Iodine No.									
			Weight.	Total lipoid. (100 B) (A)	C	D	Total lipoid. (100 D) (A)	Melt- ing point.	Weight as but- tyric acid. (100 E)	Total fatty acids. (100 E) (F)	Weight. (D+E)	Total lipoid. (100-C)	Weight.	Non- volatile fatty acids. (100 G) (D)	Melt- ing point.	Weight.	Non- volatile fatty acids. (100 H) (D)												
																		B	gm.		per cent	E	per cent	F	gm.	per cent	G	gm.	per cent
	kg.	gm.	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent											
2	3.81	1.627	0.409	25.1	0.224	13.8	39	0.995	82.5	1.219	74.9	0.071	31.25	53	0.151	67.5	65.5	0.151	67.5										
3	4.45	4.03	0.408	10.12	0.444	11.05	38.5	3.18	88.1	3.624	89.88	0.147	33.11	50	0.266	60.3	80.0	0.266	60.3										
4	3.96	5.80	0.513	8.83	0.535	9.22	39	4.74	90.0	5.275	91.17	0.186	34.89	51	0.337	63.6	79.6	0.337	63.6										
8	3.95	5.132	0.648	12.15	0.930	18.12	39	3.53	79.2	4.46	87.85	0.346	37.20	50	0.532	57.6	81.5	0.532	57.6										
age....	4.04	4.124	0.494	14.05	0.533	13.05	38.9	3.11	86.6	3.644	85.95	0.162	34.11	51	0.321	61.5	76.6	0.321	61.5										

TABLE V.

Olive Oil Diet (Dogs).

Dog No.	Weight. kg.	Total lipid.		Unsat. lipid.				Non-volatile fatty acids.				Volatile fatty acids.		Total fatty acids.		Solid fatty acids.				Liquid fatty acids.		Iodine No.
		A	gm.	Weight.		Total lipid. (100 B) (A)	C	D	Weight.	Total lipid. (100 D) (A)	Melt- ing point.	Weight as butyric acid. (100 E) (F)	E	Total fatty acids. (D+E) (100-G)	F	Weight. (100 G) (D)	Non-volatile fatty acids. (100 G) (D)	Melt- ing point.	Weight.	Non-volatile fatty acids. (100 H) (D)		
				B	gm.																per cent	
23-7	13.8	6.848	2.283	33.42	1.756	25.65	32	2.070	54.10	3.826	66.58	0.261	14.88	50	1.218	69.4	97					
23-2	12.25	5.276	1.464	27.80	2.294	43.50	40	1.465	38.97	3.759	72.20	0.408	17.80	49.5	1.511	66.0	96					
24-8	5.42	5.336	1.504	28.20	2.566	48.20	38	1.262	32.99	3.828	71.8	0.442	17.20	49	1.561	60.9	95.5					
24-9	7.06	4.531	1.526	33.70	2.046	45.30		0.895	30.83	2.941	66.3	0.465	22.70	50	1.476	72.0	84.2					
verage....	9.63	5.505	1.695	30.78	2.165	40.66	36.7	1.423	39.22	3.588	69.22	0.394	18.14	49.6	1.441	67.1	93.2					

TABLE VI.
Fat-Free Diet (Dogs).

Dog No.	Weight.	Total lipoid.	Unsataponifiable.				Non-volatile fatty acids.				Volatile fatty acids.			Total fatty acids.			Solid fatty acids.				Liquid fatty acids.		Iodine No.
			Weight.		Total lipoid. (100 B) (A)	Total lipoid. (100 D) (A)	Melt- ing point.	Weight as but- tyric acid. (100 E)	Total fatty acids. (100 F)	Weight. (D+E) (100-G)	Total lipoid. (100-G)	Weight.	Non-volatile fatty acids. (100 G)	Melt- ing point.	Weight.	Non-volatile fatty acids. (100 H)							
			A	B													C	D	E	F	G	H	
			gm.	gm.	per cent	gm.	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent					
23-7	13.6	3.465	0.582	16.80	0.851	24.63	34	1.95	69.2	2.801	83.2	0.249	29.2	51	0.555	65.1	101						
23-2	12.5	4.590	0.897	19.55	1.706	37.20	38	1.738	50.5	3.444	80.45	0.392	22.0	51	1.147	67.0	92.5						
24-9	6.16	3.502	1.012	28.90	1.402	40.00	36	0.994	41.5	2.396	71.1	0.406	29.0	50	0.930	66.3	102.5						
24-10	5.88	2.702	0.683	25.20	1.093	40.50	40	0.881	44.5	1.974	74.8	0.312	28.5	50	0.684	62.5	103						
Average....	9.53	3.505	0.793	22.61	1.263	35.58	37	1.303	51.4	2.566	77.3	0.340	27.19	50.5	0.829	65.22	100						

TABLE VII.
Fasting Dogs (Sham-Feeding).

Dog No.	Weight. kg.	Total lipid.		Unsatifiable.		Non-volatile fatty acids.			Volatile fatty acids.		Total fatty acids.		Solid fatty acids.			Liquid fatty acids.		Iodine No.
		A	Weight. (100 B) (A)	B	C	D	Total lipid. (100 D) (A)	Melt- ing point.	Weight as butyric acid.	Total fatty acid. (100 E) (F)	Weight. (D+E) (100-G)	F	G.	Non-volatile fatty acid. (100 G) (D)	Melt- ing point.	H	Non-volatile fatty acid. (100 H) (D)	
22-7	12.9 (10.9)*	1.580	0.485	30.7		0.469	29.7	37	0.607	56.41	1.076	69.3	0.099	21.7	48	0.415	88.5	79.2
23-2	13.15 (11.5)	1.283	0.242	18.9		0.302	23.6	36	0.731	70.76	1.033	81.1	0.025	8.28	49	0.298	98.5	103
24-8	5.1 (4.24)	1.277	0.431	33.8		0.362	28.4	36	0.479	55.76	0.841	66.2	0.092	25.4	48	0.253	70.0	102
24-9	6.25 (5.14)	1.284	0.525	40.9		0.284	22.1	37	0.475	62.58	0.759	59.1	0.049	17.2	49	0.241	85.0	78.6
24-10	7.45 (5.81)	2.650	0.770	29.1		1.359	51.2	41	0.475	25.84	1.834	61.9	0.514	37.9	52	0.786	58.0	83
24-11	8.52 (7.64)	2.161	0.834	38.5		0.762	35.2	37	0.553	42.06	1.315	61.5	0.213	28.0	49	0.545	71.5	97
	8.83 (7.54)	1.706	0.548	31.9		0.589	31.7	37	0.553	52.23	1.142	66.5	0.165	22.9	49	0.423	78.6	90.4

* Weights of animals at end of fasting period.

TABLE VIII.
Coconut Oil Diet (Dogs).

Dog No.	Weight, kg.	Total lipid.	Unsatifiable.			Non-volatile fatty acids.			Volatile fatty acids.			Total fatty acids.			Solid fatty acids.			Liquid fatty acids.			Iodine No.
			Total lipid. (100 B)		Weight. (100 E)	Total lipid. (100 D)		Weight as butyric acid. (100 F)	Total fatty acids. (100 G)		Weight. (100 H)	Non-volatile fatty acids. (100 I)		Melt- ing point.	Weight.		Non-volatile fatty acids. (100 J)				
			A	B		C	D		E	F		G	H		I	J					
			gm.	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent		
23-7	11.3	5.907	1.197	20.28	2.937	49.72	34	1.690	36.52	4.627	79.74	1.273	43.41	45	1.534	52.23	1.021	47.73			
23-2	12.1	3.685	0.699	18.96	2.139	58.04	37.5	0.880	29.13	3.019	82.04	0.942	44.03	47	0.853	53.0	0.864	47.0			
24-8	4.8	3.086	0.643	20.83	1.290	41.82	37	1.131	46.73	2.421	79.17	0.567	44.0	44	0.983	53.0	0.884	47.0			
24-9	5.4	4.180	0.778	18.60	1.842	44.20	38	1.548	45.66	3.390	81.40	0.978	53.0	47	0.884	47.0	0.835	54.4			
24-10	6.78	3.386	0.715	21.10	1.538	45.50	38	1.09	41.32	2.638	78.90	0.682	44.5	48	0.835	54.4	1.284	54.2			
24-11	8.1	5.056	1.126	22.20	2.368	46.89	35	1.805	43.25	4.173	77.80	0.768	32.4	46	1.284	54.2					
Average....	8.1	4.216	0.859	20.30	1.931	47.6	36.6	1.357	40.43	3.343	79.90	0.868	43.6	46	1.037	51.5				82	

TABLE IX.
Recapitulation of Averages.

	Unexaponifiable.			Non-volatile fatty acids.			Volatile fatty acids.		Total fatty acids.		Solid fatty acids.			Liquid fatty acids.		Iodine No.								
	Total lipid.	Total lipid.		Weight.	Total lipid.	Melt- ing point.	Weight as butyric acid.	Total fatty acids.	Weight. (D+E)	Total lipid. (100-C)	Weight.	Non- volatile fatty acids.	Melt- ing point.	Weight.	Non- volatile fatty acids.									
		(A)	(100 B)														(A)	(100 D)	(E)	(F)	(G)	(D)	(H)	(100 H)
	A	B	C	D	(A)	(100 D)	E	(F)	Weight. (D+E)	Total lipid. (100-C)	G	(D)	(D)	H	(D)									
	gm.	gm.	per cent	gm.	per cent			per cent	gm.	per cent	gm.	per cent		gm.	per cent									
Normal (oats) . . .	3.775	0.696	18.70	2.03	51.9	40	0.111	6.3	2.25	81.0	0.652	25.9	54.9	1.187	64.6	79.8								
Coconut oil (cats)	4.124	0.494	14.05	0.533	13.05	38.9	3.11	86.6	3.644	85.9	0.162	34.11	51	0.321	61.5	76.6								
Fat-free (cats) . . .	3.399	0.278	8.47	1.73	58.3		0.253	12.41	2.44	91.53	0.479	26.30	49.6	1.365	78.9	71.8								
Sham-feeding (oats) fasting . . .	2.043	0.800	38.71	1.07	50.25	39.7	0.028	2.67	1.097	62.71	0.328	30.6	52.4	0.699	65.1	68.8								
Coconut oil (dogs).	4.216	0.859	20.31	1.986	47.6	36.6	1.357	40.43	3.343	79.90	0.868	43.6	46	1.037	51.5	82								
Fat-free (dogs) . . .	3.565	0.793	22.61	1.263	35.58	37	1.303	51.4	2.566	77.39	0.340	27.19	50.5	0.829	65.22	100								
Sham-feeding (dogs) fasting . . .	1.706	0.548	31.9	0.589	31.7	37	0.553	52.23	1.142	68.8	0.165	22.9	49	0.423	78.6	90.4								
Olive oil (dogs) . . .	5.505	1.695	50.78	2.165	40.66	36.7	1.423	39.22	3.588	69.22	0.394	18.14	49.6	1.441	67.1	93.2								

The combustion mixture of the syrupy material was made by heating until it could be poured and then putting a layer of Na_2O_2 , NaNO_3 , and sugar in the bomb, adding a drop or two

TABLE X.
Liquid Acids.

Diet.	Method.	Weight of acids separated.	Solid and oleic fraction.	Iodine number.	Unsaturated fraction.	Bromo acids in-soluble in ether.	Bromine.	Bromo acids in-soluble in ether.	Bromine.
		gm.			gm.	gm.	per cent	gm.	per cent
Olive oil.	Ba soap-benzene.	5.167	1.257	62.5	3.324	1.756	63.59	4.287	36.02
Olive oil.	From I_2 . No deter- mination.	0.874				0.0145		1.108	32.67
Fat-free.	Ba soap-benzene.	2.564	0.311	46.6	1.322	0.1506	64.94	1.807	34.46
Fat-free.	From I_2 . No deter- mination.	1.314				0.0536	48.19	1.163	30.21
Fasting.	From I_2 . No deter- mination.	2.538				0.0526	65.35	3.296	30.13
Coconut oil.	Ba soap-benzene.	6.233	2.109	39.8	2.933	0.1762	62.74	4.286	32.70

Bromine in Various Bromo Acids.

	per cent
$\text{C}_{18}\text{H}_{33}\text{Br}_2\text{O}_2$	63.32
$\text{C}_{18}\text{H}_{31}\text{Br}_2\text{O}_2$	53.33
$\text{C}_{16}\text{H}_{29}\text{Br}_2\text{O}_2$	65.75
$\text{C}_{18}\text{H}_{33}\text{Br}_2\text{O}_2$	55.94
$\text{C}_{18}\text{H}_{31}\text{Br}_2\text{O}_2$	36.19
$\text{C}_{16}\text{H}_{29}\text{Br}_2\text{O}_2$	38.64

of the bromide, covering with another layer of Na_2O_2 , etc., and finally shaking thoroughly. The results of this work are tabulated in Table X. The data from the feeding experiments are contained in Tables I to IX.

Analysis of Data.—As pointed out above in the work with cats on normal, fat-free, and sham-feeding diets probably most of the volatile acid was lost in evaporating to dryness before the total lipid was weighed. The very large amount of volatile acid obtained from the coconut oil diet indicates either that the cats normally excrete a large amount of volatile acids or they do not handle well the volatile acids of coconut oil. If the first of these possibilities is true, the loss of this volatile acid would have a very appreciable effect on the total lipid. Consequently, no definite conclusions can be drawn from these figures or the various percentage figures depending on them, and the greater weight in analysis and interpretation will therefore be given to the later experiments and especially to those with dogs. The unsaponifiable fraction and the solid and liquid fatty acid fraction should not, however, be affected by this loss of volatile acid. It may be noted here that the proximate analysis of the feces fat differs markedly from that of the food fat.

Total Lipoid.—On a fat-free diet there is but slight decrease in total lipid from the amount excreted in a high fat diet.

On a sham-feeding diet there is a marked decrease in the total lipid, but still an appreciable amount is excreted.

The excretion on an olive oil diet is considerably higher than on a coconut oil diet, but this is due almost entirely to an increased value of the unsaponifiable fraction. The non-volatile and volatile fatty acid fractions are only slightly greater.

There appears to be no relationship between the weight of the animal and the amount of lipid extracted.

Even with the probable loss of considerable volatile acid the cats excrete far more lipid per unit body weight than the dogs. In this connection attention is directed to the fact noted above that the cat feces tended to be more fluid than those of the dogs which would make possible a sweeping out of some material which might otherwise have been absorbed. The fact that the lipid excretion is greater per kilo for cats even in fasting and on the fat-free diet indicates that this lipid does not necessarily arise from the food.

Unsaponifiable Fraction.—In the case of the cats the unsaponifiable fraction is highest on a sham-feeding diet, lowest on a fat-free, and intermediate on the normal and coconut oil diets,

indicating that an important source of unsaponifiable matter is tissue destruction which would be increased in fasting. It is noticeable that nearly as much unsaponifiable matter is excreted on a coconut oil diet as on a normal diet while the non-volatile fatty acid fraction is four times on a normal what it is on a coconut oil diet.

In the dogs the amount of the unsaponifiable fraction varies as the total lipid. The percentage amount is higher in the sham-feeding and olive oil diets than in the other two.

Holmes and Kerr (2) have reported that the ether-soluble unsaponifiable matter of human feces consisted largely of coprosterol, indicating that the origin of this fraction was the sterols of the food. No examination of this material was made in the present work, but it is hoped to report on it later.

Non-Volatile Fatty Acids.—The non-volatile fatty acid fraction varies as the total lipid; but there is almost as much excreted on a coconut oil as on an olive oil diet. This makes the percentage amount higher on the coconut oil diet. It is low on sham-feeding.

The melting points of this fraction are remarkably constant at about body temperature. The one exception (fat-free diet in cats) where the material remained liquid may be due to a larger percentage of volatile acid remaining in this fraction. The similar fraction of fatty acids from the blood melts at about the same point (6) which leads to the suggestion that since the solid acids and their esters are difficult to transport because of their high melting point, the amount of solid acid present in these mixtures is about the maximum that can be carried at body temperature without solidification.

Volatile Fatty Acids.—The volatile acid fraction varies also as the total lipid; but, in the dog, there is very little difference between the fat-free, coconut oil, and olive oil diets. The percentage is high on the fat-free and low on the olive oil diet. In the case of some dogs the volatile acid is highest on a fat-free diet. The large amount of volatile acid from the coconut oil diet in cats is remarkable, being over twice the excretion from any of the dogs. It was the only cat experiment run by the corrected method used on the dog runs and is, therefore, comparable to the latter.

Total Fatty Acids.—The total fatty acids follow the same order in amount excreted as the total lipid. The percentage amounts

in the olive oil and sham-feeding diets are low due to the higher percentage of unsaponifiable substance.

Solid Fatty Acids.—The solid acids in dogs follow the same order as total lipid except that they are much higher on a coconut oil diet and much lower on an olive oil diet. The percentage of total lipid is also much the highest on a coconut oil diet and lowest on an olive oil diet.

The melting points of this solid fraction are fairly constant, averaging about 50°C. The melting points of the same fraction from the blood average about 55°C. (6).

Liquid Fatty Acids.—The order in the amounts of the liquid acids excreted is the same as in total lipid, the highest being excreted on an olive oil diet. The percentage, liquid fatty acids of total lipid, is almost constant, but somewhat higher on an olive oil diet.

The ratio of liquid acid to solid acid in the non-volatile acid fraction lies between two and three in all experiments on both cats and dogs, except the two coconut oil diets, where it is less than two (a little above one in the case of the dogs) and in the olive oil and sham-feeding experiments in dogs where it is more than three. (The ratio of liquid to solid acids in the blood averages a little over three (6)). The high ratio in the fasting experiment may be due to experimental error in the separation of the small amounts of acid found here. The high ratio on the olive oil diet is the result of a low excretion of solid acids and a somewhat higher excretion of liquid acids, while the low ratio on the coconut oil diet is due to a slightly lower liquid acid excretion and a much higher solid acid excretion. The diet seems to show its effect much more on the solid acids than on the liquid acids, due to the possible greater utilizability of the liquid acids.

The iodine numbers of the liquid acids are fairly constant between 70 and 100 and appear to bear no relationship to the fat of the food.

The liquid fatty acid fraction was separated into its constituent parts by the barium soap-benzene method described above. The results of the division are given in Table X. As may be seen, the separation and analysis indicate that the fraction consists mainly of oleic acid with a small amount (generally less than 10 per cent) of C₁₈ or C₁₆ acids containing three double bonds. The

fact that any amount of highly unsaturated acid should escape oxidation during the long series of operations necessary for its isolation is remarkable and indicates that the amount of these unsaturated acids is probably much larger in the fresh feces.

DISCUSSION.

There are at least four sources of fat in feces: intestinal bacteria, desquamated intestinal cells, unabsorbed lipid residues from the food, and excretions from the blood either directly or indirectly through the bile and other digestive secretions.

Nothing is known concerning the lipid content of intestinal bacteria or of intestinal cells, but the assumption that feces fat comes from these materials would perhaps explain the lack of relationship between the weight of the animal and the amount of fat excreted, since these factors would be expected to act irregularly. On a sham-feeding diet the desquamation of intestinal cells would be expected to be nearly as great as on a nutritive ration due to the mechanical action of the agar and bone ash, but the bacterial products would be expected to be less, due to lack of material on which the bacteria could work. It is probable also that there would not be a normal flow of digestive secretions, even though the mechanical stimulus of material in the intestine is present. On this sham-feeding the total lipid is very low although the relations between the lipid constituents are not markedly different from those on the diets.

In confirmation of the work of Hill and Bloor (1) and Holmes and Kerr (2) which gives evidence against the source of feces fat in unabsorbed food residues, may be listed the high excretion on a fat-free diet, the appreciable excretion on a fasting diet, and the lack of similarity in composition between the food fat and feces fat. On the other hand, the food exercises some influence on the feces fat as is evidenced by the low excretion by fasting animals, the high excretion of solid acids on a diet high in solid acids, the increased excretion of liquid acids on a diet high in liquid acids, and possibly the high excretion of unsaponifiable material on an olive oil diet, due to unsaponifiable material in the olive oil. These phenomena may, however, be explained on another basis, as will be shown below.

The most probable source of feces fat seems to be excretion from the blood either directly or indirectly as is evidenced by similarity of the fecal lipoids to those of plasma in the ratios of liquid to solid acids in the non-volatile fraction, in the melting points of the non-volatile fraction and its solid component, and in the relatively constant composition of the liquid acid fraction. The iodine numbers of the liquid fraction from feces are lower than those of the blood, but this may be due to delay and exposure to oxidation incident to collection. The intestine appears to have some reducing power as is shown by the reduction of cholesterol to coprosterol (2) and this may account for the saturation of some of the double bonds.

If we assume that feces fat originates in excretion from the blood, there are two possible ways in which it may occur. It may be a true excretion of unusable material—waste fat from lipid metabolism—in which case, we would expect the fat excreted to reflect in kind the fat metabolized; for example, a high excretion of solid acids on a diet high in solid acids. The low excretion in fasting would be explained as an attempt by the organism to conserve its resources by burning lipid which on an adequate diet it would excrete.

On the other hand, the excretion may be considered as a leakage of usable fat due to fat plethora analogous to the leakage of amino acids through the kidney or the alimentary glycosuria which occurs on high sugar intake. If this is true, we would expect the lipid excreted to reflect in kind the lipid carried by the blood, and although we have no data bearing directly on the question we would expect the lipid of the blood, during absorption at least, and probably at all times, to be similar to, or at least to be influenced by, the lipid of the diet. This again would explain the points in which the feces fat appears to be affected by the fat of the diet. This conception might explain too the lack of any relationship between the weight of the animal and the amount of fat excreted, since such a leakage might be expected to depend on the nutritive condition of the animal and the permeability of the intestinal tract and so to vary with different animals and from time to time.

The excretion of unsaponifiable material by cats calls for comment. The fact that it is highest on a sham-feeding diet points

to the origin of part of it at least in tissue destruction. In dogs, however, this is not so evident, although the high percentage in sham-feeding indicates a similar condition. The unsaponifiable material is also high in percentage on an olive oil diet, but this may be due to the influence of unsaponifiable material in the oil. The high percentage of unsaponifiable material on these two diets tends to lower the percentage of the various fatty acid fractions throughout.

The high excretion of volatile acids by cats on a coconut oil diet and the attendant decrease in non-volatile acids may show that the cats cannot handle the coconut oil well. The dogs appear to assimilate it as well as olive oil since there is no increase in volatile acid even though the diet contains a much higher percentage of coconut oil. In fact, the volatile acids in dogs are remarkably constant except on a fasting diet. The highest percentage excretion is on a fat-free diet containing large amounts of carbohydrate and may possibly be explained by butyric acid fermentation of the carbohydrate by bacteria. The work is being continued.

SUMMARY AND CONCLUSIONS.

Feces "fat" from cats and dogs, fasting and on standard diets, has been separated into fractions and the composition of these fractions studied with special reference to the source of the fatty material.

That it does not arise directly from unabsorbed fatty material from the food is shown by the following facts. In many cases almost as much fatty material appears in the feces on a fat-free as on a fat diet. There is a considerable output in fasting which is similar in properties and in the relations of its components to that excreted when food was given. The composition of the food fat is different from that of the feces fat.

The influence of fat in the diet is, however, shown by the increased excretion of the solid fatty acids on a diet high in solid acids and a similar increase of liquid acid excretion when the food contains large amounts of liquid fatty acids. The effects of food is shown by the much greater lipoid excretion on a fat-free diet over that on fasting.

There is a marked similarity between the blood and fecal lipid with regard to the ratios of solid to liquid fatty acids and the melting points of the non-volatile fatty acid fraction and its solid component which makes it probable that the fatty material of the feces has its origin largely in the blood.

No definite relation between the weight of the animal and the amount of excreted lipid could be demonstrated.

The presence in feces of small amounts of an eighteen carbon fatty acid with three double bonds was shown.

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THE DETERMINATION OF IODINE IN FOOD, DRINK, AND EXCRETA.

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The older determinations on minute quantities of iodine may be divided into two classes: those that gave too high results, and those that gave negative results. There is certainly room for improvement in methods, but the method described in this paper is believed to be the most accurate now known. The possible error depends on the percentage of iodine. The adequacy of the method is based on two criteria: first, the yield is in direct proportion to the size of the sample, and second, iodine added in inorganic form is recovered.

This work was done entirely independently of that of von Fellenberg who designed a somewhat similar method.¹ It is significant also that von Fellenberg had difficulty with losses of iodine in ashing:

"Seit Beginn meiner Arbeiten sehe ich die grossen Schwierigkeiten, die darin liegen, die organischen Substanzen vollständig zu verbrennen ohne dabei Jod zu verlieren. Die Verbrennung in einem Rohr schwebte mir auch als Ideal vor; . . . Nun sehe ich, dass Sie das Problem gelöst haben. Wenn ich dazu komme, werde ich gerne nach Ihrer Methode Versuche unternehmen.

"Ich suchte den Jodverlust bis jetzt zu vermeiden durch verhältnismässig grosse Zusätze von Pottasche. Dadurch wird aber die Verbrennung erschwert, so dass man die Kohle meist noch zwei mal mit Wasser ausziehen muss, um sie ganz zu veraschen.

"Bei den meisten Substanzen scheint mir diese Methode ziemlich sicher zu sein; bei NaCl-haltigen Materialien, wie Harn, sind die Verluste schwerer zu vermeiden." (Private communication from von Fellenberg.)

¹ von Fellenberg, T., Untersuchung über das Vorkommen von Jod in der Natur, *Mitt. Gebiete der Lebensmitteluntersuch. u. Hyg.*, 1923, xiv, 161.

Some points on the difficulties of ashing may be considered. Sørensen observed that the total solids of sea water could not be estimated by the method of "ashing." In 1908 I observed that on the evaporation of sea water, certain secondary reactions took place. These observations were extended² in 1916, but perhaps all that it is necessary to mention here is that when sea water is evaporated nearly to dryness, hydrochloric acid is given off, although at the beginning of evaporation in these experiments the pH was 8.2 and this increased in value for a considerable period. Since hydrochloric acid is given off, it is probable that at a certain stage the pH is just right for the reaction between iodate and iodide to liberate iodine, according to the reaction $\text{IO}_3^- + 6\text{H}^+ + 5\text{I}^- = 3\text{I}_2 + 3\text{H}_2\text{O}$. Furthermore, if any excess iodide (which is improbable) remained, some of it might be evolved as hydriodic acid; any remaining as iodate would be retained completely in the salt if the temperature did not exceed 110°. In evaporating sea water to retain all of its constituents, it was not evaporated to dryness, but was bottled in the wet stage. In 1920 it was pointed out that iodine in sea water might be utilized for the nutrition of the thyroid gland.^{3,4} It must be admitted, however, that the method described retained only part of the iodine in sea water as iodate. A better method was described in 1922, by the addition of enough soda to precipitate all the calcium and magnesium as carbonates before evaporation.⁵ This method was checked by determinations of iodine in sea water, and the iodine in the salt.

In the presence of much organic matter in a sample for iodine determination, however, large quantities of CO_2 and other organic acids are produced in the ashing, and it is very difficult to prevent entirely the loss of iodine merely by the time-honored method of adding alkali before ashing. Furthermore, this alkali interferes with the ashing process, and since in many foodstuffs it is required to use a sample weighing several kilos in order to ob-

² McClendon, J. F., Gault, C. E., and Mulholland, S., The hydrogen ion concentration, CO_2 tension, and CO_2 content of sea-water, *Carnegie Inst. Washington, Pub. 251*, 1917, 39.

³ McClendon, J. F., *J. Biol. Chem.*, 1921, xlv, p. xxvii.

⁴ McClendon, J. F., *Science*, 1922, lv, 358.

⁵ McClendon, J. F., *Science*, 1922, lvi, 269.

tain enough iodine for a quantitative analysis, the process of ashing with large additions of alkali is very tedious and expensive. Kendall⁶ greatly improved the technique of determining iodine in organic matter, and his method was tried on cereals,⁷ but it was found that the iodine was too low for Kendall's method, since if the size of the sample was increased, the size of the yield was not increased in proportion. A method was tried to reduce the content of organic matter by the addition of yeast, then driving off the water after making the sample alkaline, and in the combustion passing the fumes through an alkaline solution to catch the iodine.⁷ Later developments have omitted the fermentation but depended on the alkaline solution to prevent loss in ashing. Many methods of ashing were tried. It was found that samples containing relatively large amounts of iodine could be ashed in the bomb of a calorimeter. In increasing the size of the sample, however, up to about 25 gm., the ignition plug of the bomb blew out. If the organic matter was small in amount as in water residues, it would not burn in the bomb. A very heavy steel bomb was made and heated from the outside to burn water residues.⁸ A number of analyses were made in this way, using a copper-asbestos gasket to prevent the escape of iodine during the heating. This method, however, was given up. Cereals were destructively distilled in a steel retort, passing the gas through an alkaline solution, but the difficulty in ashing the remainder was almost as great as ashing the whole sample. Therefore, the following procedure was adopted as the final method.⁹⁻¹⁴

⁶ Kendall, E. C., *J. Biol. Chem.*, 1914, xix, 251.

⁷ McClendon, J. F., and Rask, O. S., *Proc. Soc. Exp. Biol. and Med.*, 1922-23, xx, 101.

⁸ McClendon, J. F., *J. Biol. Chem.*, 1923, lv, p. xvi.

⁹ McClendon, J. F., and Williams, A., *Proc. Soc. Exp. Biol. and Med.*, 1922-23, xx, 286.

¹⁰ McClendon, J. F., and Williams, A., *J. Am. Med. Assn.*, 1923, lxxx, 600.

¹¹ McClendon, J. F., *Proc. Soc. Exp. Biol. and Med.*, 1922-23, xx, 351.

¹² McClendon, J. F., *Science*, 1924, lix, 21.

¹³ McClendon, J. F., and Hathaway, J. C., *Proc. Exp. Biol. and Med.*, 1923-24, xxi, 129.

¹⁴ McClendon, J. F., Hathaway, J. C., and Netz, L., *Proc. Soc. Exp. Biol. and Med.*, 1923-24, xxi, 347.

Method of Iodine Determination in Water.

The unique characteristic of this method is the use of the combustion tube and micro colorimeter. In case of water samples it is necessary to use at least 100 liters unless the iodine content is high. This is made alkaline with the addition of sodium carbonate, sodium bicarbonate, or sodium hydroxide, and it must remain alkaline to phenol red paper during the entire period of evaporation. The easiest method of evaporation is to insert a 1 inch pipe into a barrel, place the water in the barrel and lead the pipe down into a large dish-pan into which 2 gm. of sodium bicarbonate have been placed. By means of a very large gas flame, or other heater, evaporation is quickly effected. It is not probable that any iodine is acquired from the gas since practically all the iodine in coal remains in the coke and in the ammoniacal liquids of the gas works. If any escape these, they would be caught in the lime over which the gas is passed in purification. Since ordinary coal gas contains some water gas, the carbon monoxide should reduce any iodine to iodide and prevent it from evolving in a vapor state. Another convenient method of evaporation is as follows: A zinc tank in which 100 liters could be accurately measured was rigged up with a float valve to deliver the water to the dish-pan, and attached to this float valve was a trigger to turn off the gas when the evaporation was completed. By this method the water is evaporated to about 1 liter. A considerable amount of carbonates of alkaline earths are incrusting in the pan or precipitated to the bottom. These are filtered off as it was found they never contained more than 2 per cent of the iodine. The sample is then evaporated to dryness, powdered, and placed in the boat, *D* (Fig. 1). This boat is best made of silica, but a sheet of nickel or even iron can be used. The boat is inserted into the silica tube, *C*. (A Pyrex tube may be substituted for this silica tube, but is not so convenient.) 10 cc. of a 10 per cent sodium hydroxide solution are placed in the Pyrex test-tube, *B*. The end, *A*, of the silica tube is inserted into this sodium hydroxide solution. The water-cooled stopper, *F*, closes the other end of the silica tube. Through a glass tube passing through the stopper, *F*, a stream of oxygen is passed over the sample and out through the sodium hydroxide solu-

tion. The silica tube is heated to dull redness for the shortest length of time required to burn all the organic matter in the sample. This varied with the depth of the layer of powder and the

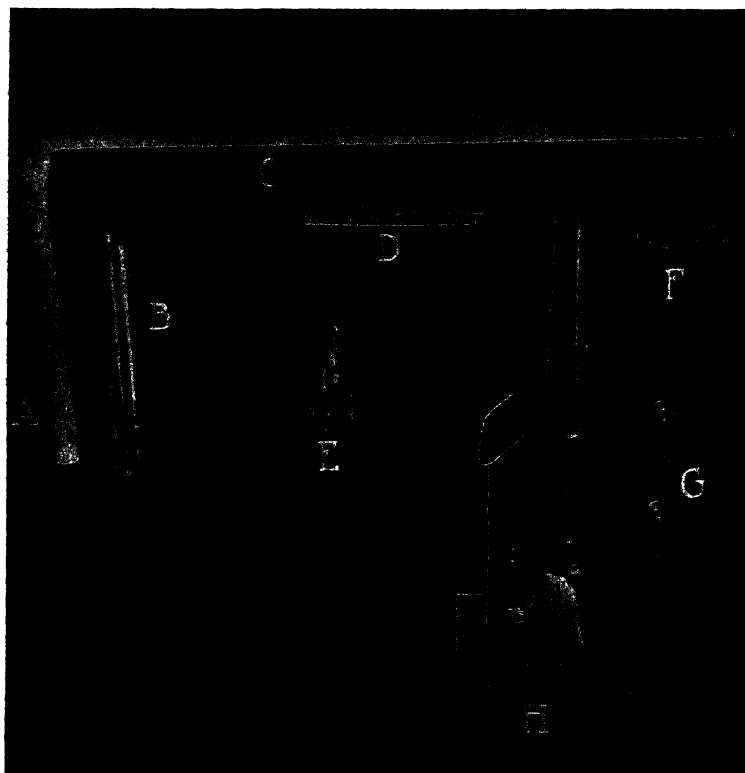


FIG. 1. Apparatus for determining iodine in water residues.

A-C = Silica combustion tube.

B = Pyrex tube containing alkaline absorption solution.

D = Combustion boat.

E = 12 cc. separatory funnel.

F = Water-cooled rubber stopper admitting oxygen into silica tube.

G = Colorimeter cups holding 1 cc. at 2 cm. depth.

H = Bausch and Lomb micro colorimeter.

proportion of organic matter in water. If on removing it from the tube it is not completely burned, it can be reinserted and the operation repeated. In case the water cooling was not ef-

fectured on the rubber stopper, it sometimes caught fire. The water cooling device is simply a piece of tin or lead pipe 5 mm. in diameter inserted through one hole, looped around and passing out another hole in the rubber stopper. The passing of cold water through this tube shielded the stopper from the great heat. This shielding is not necessary in the ordinary combustion tube, but by means of this water cooling device it is possible to use tubes of much larger diameter and shorter in length, therefore more easily handled. The ash is powdered, and the sodium hydroxide solution together with the rinsings of the tube are evaporated to dryness and powdered and then both mixed in the mortar. A measured portion of water (for instance, 15 cc.) is added and the powder ground in the water so as to extract the iodides and iodates. This is filtered and an accurately measured aliquot taken (for instance, 7.5 cc.). This aliquot is neutralized with concentrated hydrochloric acid and the volume made up to 10 cc. It is then placed in the 12 cc. separatory funnel, *E* (Fig. 1), and 1 drop of concentrated hydrochloric acid and 1 cc. of purified carbon tetrachloride are added and the funnel is shaken. If any pink color appears it denotes that iodate as well as iodide is present in the ash. 1 drop of 0.1*N* arsenous acid is added and allowed to remain for 20 minutes. This is to reduce any excess iodate that might be present. 1 drop of nitrosyl sulfuric acid is added to oxidize iodide to iodine and the separatory funnel shaken hard for 2 minutes to extract the iodine. The carbon tetrachloride is allowed to run into a glass-stoppered bottle and centrifuged for 1 minute to remove any water droplets. It is then transferred to one cup of the Bausch and Lomb micro colorimeter, *H*, and into the other cup is placed carbon tetrachloride, 1 cc. of which contains 0.1 mg. of pure iodine. This micro colorimeter has the advantage of measuring very small amounts of iodine, since 1 cc. of carbon tetrachloride fills the cup to the depth of 2 cm. The iodine extracted is determined colorimetrically and another cubic centimeter of carbon tetrachloride added to the separatory funnel and another extraction made and determined in the colorimeter. By repeated extractions practically all of the iodine may be recovered and measured and the results of the different extractions added together to determine the amount in the aliquot, and knowing the relative volume of the

aliquot, the iodine in the total sample may be calculated. It was possible to obtain a fair degree of accuracy with a single extraction on the assumption that about 80 per cent of the iodine in the separatory funnel was extracted from 10 cc. of an aqueous solution by 1 cc. of carbon tetrachloride. The partition coefficient of iodine between these two solvents varies with the electrolyte content of the water. In some cases as high as 86 to 87 per cent of the iodine was obtained in the first extraction. Under uniform conditions a factor can be determined for this partition coefficient and one extraction is all that is necessary.

It is always possible to have the water phase saturated with NaCl, and if other electrolytes are small in amount, the conditions approach uniformity.

Carbon tetrachloride contains reducing substances which must be removed. The simplest method is to take an entire drum of the commercial product, place it in a large glass vessel in the sunlight and add bromine to it as this bromine is decolorized, for several days or until it will remain for several hours without diminution in the bromine color. The excess bromine must be removed in some way as by shaking the carbon tetrachloride with an alkaline solution. After this it is washed with pure water several times or continuously by inserting a tube to the bottom and allowing pure water to bubble up through it. The water is then separated off, and plaster of Paris added to absorb the remaining water. It is then filtered through paper and distilled. It is usually found that some water and other impurities come off in the first portion of the distillate, and this is rejected. It is also necessary to stop the distillation before the end to guard against the effect of excessive heating on non-volatile impurities. Carbon tetrachloride is stored in the dark.

Iodine is purified by mixing it with KI, subliming it, then re-subliming it, and drying in a desiccator. With this iodine dissolved in carbon tetrachloride that has been treated in the above manner, the color gradually changes from a pink to a yellowish. This yellowish substance is apparently a compound of iodine and chlorine, but whatever be its nature, its development must be prevented. This is most easily done by overlaying the iodine solution in carbon tetrachloride with a layer of water containing 1 drop of nitrosyl sulfuric acid in 50 cc. and containing iodine at

the same solution tension as that in the carbon tetrachloride. If iodine in carbon tetrachloride is sealed in glass tubes, the color disappears entirely in a few minutes and cannot be brought back by shaking with oxidizing or reducing solutions, but if after placing the carbon tetrachloride solution in the tube, it is overlaid, as is directed above, with an aqueous solution, the sealing can be effected without bleaching of the iodine. The reason for this is probably that the vapor of carbon tetrachloride is overheated during the sealing and the reaction products react with



FIG. 2. Apparatus for combustion of large quantities of organic matter preliminary to iodine determination.

A = Water-cooled rubber stopper.

B = Silica combustion tube, 10 cm. bore.

C = Absorption apparatus containing alkaline solution.

D = Cooling coil.

the iodine, whereas the overlying aqueous solution prevents the evaporation of carbon tetrachloride and prevents any of its vapor from being overheated. Directions for preparing the reagents may be found in Treadwell and Hall.¹⁵

Determination of Iodine in the Presence of Large Quantities of Organic Matter.

This method is the same as that described above with the exception of the first ashing process. It is sometimes necessary to use several kilos of foodstuff in order to obtain 0.01 mg. of

¹⁵ Treadwell, F. P., and Hall, W. T., *Analytical chemistry. Qualitative analysis*, New York and London, 5th edition, 1921, i, 350, 369; *Analytical chemistry. Quantitative analysis*, New York and London, 4th edition, 1915, ii, 646, 651.

iodine. Food is placed on a piece of sheet iron in the large silica tube, *B* (Fig. 2). This tube is 10 cm. bore and more than a meter long. The length, however, could be greatly reduced. In the smaller end of this silica tube is inserted a double coil of tin or lead pipe, *D*, through which cold water is circulated. 1 liter of 10 per cent NaOH solution is placed in the Pyrex absorption vessel, *C*, and this is adapted over the downwardly directed end of the silica tube containing the cooling coil. It is practically necessary to have a notch or separate opening at the top of the absorption vessel, *C*, through which the cooling tubes can pass. A separate opening can be made air-tight by means of a double bored rubber stopper which is either passed downward over the free ends of the cooling tubes or is fitted around them by splitting it. The opening between the silica tube and the vessel, *C*, is closed by means of a wet mass of asbestos fibers. By means of a side neck on the vessel, *C*, connected with a rotary exhaust pump, a very rapid air current is sucked out of the silica tube and through the alkali solution. At the same time, cold water is passed through the cooling coil. Oxygen is allowed to spurt into the open end of the tube, *B*. By means of a gas flame the material in the tube, *B*, is ignited. The combustion is always very rapid and it is necessary to use a great deal of oxygen in order to accomplish complete combustion. This necessitates a very rapid action of the air pump in order to draw out all the products of combustion through the alkaline solution. It is then difficult to get complete absorption and it is practically necessary to insert an additional absorption vessel between the vessel, *C*, and the air pump. The most rapid portion of the combustion process is usually completed within half an hour. If it is desired to slow up this process the sample may be divided and only part of it inserted at a time. In case of a liquid like cod liver oil it is impossible to get complete combustion if the whole sample is placed in the tube at once. The only practical method is to drop or spray it into the tube during the combustion.

During the earlier experiments the air pump was not used, but the large end of the silica tube was closed by means of the water-cooled rubber stopper, *A*, and the pressure of oxygen from the tank was depended on to force the products of combustion through the alkaline solution. It was necessary to ignite the sample after inserting the rubber stopper either by means

of electric wires or by means of a gas flame inserted through a little hole closed by a silica stopper. If the oxygen was not forced in with sufficient rapidity, an explosion was liable to result which would blow out the rubber stopper; therefore, the suction method was substituted. The mixing of a little air with the oxygen is not at all objectionable.

After the volatile products are consumed the carbon is burned more slowly and it is then necessary to apply external heat to the tube. If the ash is reduced to relatively small volume it is sometimes preferable to stop the combustion in this large tube and grind the ash to a powder and complete the combustion of the remaining particles of carbon in the smaller tube used for water residues. The sodium hydroxide solution and rinsings of the tube are evaporated to dryness and usually contain some unburned organic matter. This may be burned in the smaller tube used for water analysis. The remainder of the analysis is the same as that for water analysis given above except that the large volume of ash may require a larger portion of water for extraction and the aliquot transferred to the separatory funnel may necessarily be a smaller fraction of the total. It is very desirable to have a small ball mill for grinding the ash with water.

If only a few analyses are to be made it is possible to use a Pyrex combustion tube. The cracking of the Pyrex tube where the hot portion reached the alkaline solution was avoided in a number of experiments by wrapping a section of the Pyrex tube with a thin sheet of asbestos paper, then winding around it about fifteen turns of small lead pipe through which cold water was flowing during the whole process of combustion.

It is easily possible to detect 0.001 mg. of iodine in 1 cc. of carbon tetrachloride and make a roughly quantitative determination of it in the micro colorimeter. It is desirable, however, to have 0.01 mg. of iodine or a greater quantity in the sample used for analysis. Sometimes there is a slight yellowish tinge to the carbon tetrachloride used in extracting the iodine. It is sometimes possible to make a determination even in this case by transferring this carbon tetrachloride into another small separatory funnel together with 1 cc. of water and 1 drop of 0.1 N sulfurous acid. On shaking, all the iodine will pass into the sulfurous acid as iodide. The carbon tetrachloride is now withdrawn, and about one-tenth of a drop of nitrosyl sulfuric acid

added together with 1 cc. of pure carbon tetrachloride. On shaking, the iodine will now pass into the carbon tetrachloride and it may happen that the yellow color does not develop, but only the pink color of the iodine remains. Much trouble that might arise in the determination may be attributed to failure to obtain complete combustion of organic matter, and if the determination is repeated and care taken that the final alkaline solution used in absorption is free from organic matter, the analysis will usually be normal.

THE CREATINE CONTENT OF BRAIN.

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There are no systematic observations on the amount of creatine in brain. It is known to be present in that organ, for Beker (1) was able to isolate creatinine zinc chloride in characteristic form from ox brain; there are also a few scattered observations on its amount in brain. Thus, Beker gives the amount in ox brain as 51 to 63 mg. of creatinine per 100 gm. of tissue. This amount presumably refers to the hemispheres as he mentions the cerebellum and the white matter separately as containing 64 to 71 and 48 to 56 mg. of creatinine per 100 gm. of tissue, respectively. The brain of the dog, according to the same observer, contains 54 to 57 mg. of creatinine per 100 gm. of tissue. Beker's figures are undoubtedly too low as we have been able to isolate from the cerebral hemispheres of the ox 95 to 96 mg. of total creatine per 100 gm. of tissue as the relatively insoluble creatinine potassium picrate. Janney and Blatherwick (2) give the creatine content of dog brain as 110 to 124 mg. per 100 gm. Baumann and Hines (3) mention a dog brain and a beef brain as containing 120 and 113 mg. of creatine per 100 gm. of tissue, respectively.

It was partly with the idea of filling up this gap in our knowledge of the biochemistry of creatine that induced us to undertake a systematic investigation into the content of creatine in the brain of different animals. Thus it was of interest to see if the creatine content of the brains of different species varied in the same manner as the creatine content of the muscles. The effect of fasting, and of creatine feeding, on the creatine in the brain is a point also worthy of attention, when we remember the effect of these conditions on the creatine of the muscle.

Material.

The brains of the sheep, pig, and cattle were obtained from the local stock-yards. The animals were killed in the forenoon, and we received the heads during the afternoon. The skull was opened and the brains were removed, weighed, and placed in 5 N H_2SO_4 , ready for hydrolysis, before night. The dogs, cats, and rabbits were laboratory animals; the cats and rabbits being used solely for this work and were carefully fed for some days previous to death. The dogs were animals which had been used for other experimental work. In all cases the brain was removed immediately after death. Autopsy material served as our source of human brain.

Analytical Method.

The analytical method used was that of Baumann and Hines (3) for the determination of total creatine in muscle, with suitable modifications of quantities. The brain, after removal from the skull, was separated into the hemispheres and cerebellum. The gray matter of the cerebral hemispheres was separated as far as possible from the white, pulped in a mortar, and about 5 gm. were weighed by difference into a small Erlenmeyer flask; 12.5 cc. of 5 N H_2SO_4 were then added. With the brains of small animals, or in examining the different parts of a larger brain, the gray matter was weighed directly after dissection. The Erlenmeyer flask was then attached to a reflux condenser and heated in a boiling water bath for 3 hours. The contents of the flask were filtered while hot into a 25 cc. volumetric flask, the Erlenmeyer flask and the small amount of residue on the filter being washed with small quantities of boiling water until the total volume of filtrate was 25 cc. 10 cc. of this filtrate were pipetted into a 50 cc. volumetric flask, 9 cc. of 2.5 N NaOH added, the solution was cooled and diluted to the mark with saturated picric acid solution, purified to give a minimum coloration with alkali according to Folin and Doisy (4). After thoroughly mixing, the precipitated proteins were filtered off, and 25 cc. of the clear filtrate were taken for the creatinine determination. This was carried out in a 100 cc. volumetric flask; 3.0 cc. of 2.5 N NaOH were added to the 25 cc. filtrate and allowed to stand 10 minutes. It was then made up to the mark, read against the standard color similarly developed (using 15 cc. of

picric acid, water to 25 cc., and 2.5 cc. of 2.5 N NaOH), and placed at 10 mm. The standard usually contained 1 mg. of creatinine as creatinine zinc chloride, prepared according to the directions of Benedict (5) or Edgar (6). In the case of some of the cerebellum determinations where much higher values were found the standard contained 1.6 mg. of creatinine and both the standard and the determination were made to a volume of 200 cc.

We have examined the analytical method from the standpoint of the criticisms of Janney and Blatherwick and find that both acid and water extraction give the same result. We have isolated as potassium creatinine picrate 88 to 89 per cent of the expected creatine. The filtrate from the potassium creatinine picrate yielded a further 8 per cent of the expected creatine determined by the Jaffé color reaction. We do not take it that this 8 per cent of the creatine, as obtained by the Jaffé test on the potassium creatinine picrate filtrate, represents any substance other than creatine (or creatinine). Potassium creatinine picrate is not an absolutely insoluble salt; its solubility in water at 20°C. is 1 part in 554 (7). Its solubility in presence of excess of picric acid has not been determined, but it would be quite sufficient, even if the solubility coefficient were half its ordinary value, to account for a Jaffé test in the filtrate from the double salt to the extent we have found.

Creatine Content of Brain According to Species.

In Table I are collected the results on the different animals, and it will be seen at once that, just as in the amount of creatine in muscle, each species possesses an amount of creatine in brain characteristic for that animal. Thus the pig, cow, and sheep possess amounts of creatine in the hemispheres low in comparison with the rabbit, dog, cat, and man. The differences are more marked in the creatine content of the cerebellum of the different animals, man being in this respect markedly higher than the rest of the animals. The cerebellum of all animals is richer in creatine than the hemispheres. Undoubtedly the water content of the various brains is a variable factor influencing the results. We have determined at the same time the N content of the brain, and the ratio of the milligrams of creatine to 1 gm. of N is shown in Table II. With the exception of the fetal calf (and here we had only

TABLE I.

Animal.	Total creatine.		Total nitrogen.	
	Cerebral hemi-spheres.	Cerebellum.	Cerebral hemi-spheres.	Cerebellum.
	<i>mg. per 100 gm.</i>	<i>mg. per 100 gm.</i>	<i>per cent</i>	<i>per cent</i>
Pig..... 1	106.6	123.7	1.53	1.63
2	101.8	112.7	1.54	
3	106.7	114.3	1.55	1.63
4	106.7	120.3	1.52	
5	102.8	117.0	1.52	
6	104.7		1.52	
Average.....	104.9	117.6	1.53	1.63
Cow..... 1	116.0	132.5	1.74	1.74
2	113.8	138.2	1.75	1.84
3	108.4	129.3	1.58	1.74
4	101.8	120.0	1.61	1.67
5	105.8	119.6	1.74	1.72
6	99.5	126.4	1.63	1.80
7	107.0	125.5	1.55	1.64
8	101.7	117.1	1.60	1.71
9	101.1	119.0	1.66	1.85
Average.....	106.1	125.3	1.65	1.74
Calf..... 1	103.8	124.2	1.64	1.66
2	106.0	128.6	1.58	1.80
3	102.5	130.4	1.43	1.71
4	100.3	135.1	1.62	1.69
Average.....	103.1	129.6	1.57	1.71
Fetal calf..... 1	74.0	Whole brain.	0.87	Whole brain.
2	71.0	95.6	0.91	
Sheep..... 1	108.8	140.0	1.52	1.54
2	104.4	121.1	1.48	1.49
3	102.2	115.4	1.55	1.52
4	Lost.	129.2	1.48	
5	117.8	148.4	1.69	1.69
6	110.2	121.3	1.61	1.72
7	115.2	140.7	1.69	1.69
8	101.7	125.1	1.64	1.64

TABLE I—Continued.

Animal.	Total creatine.		Total nitrogen.	
	Cerebral hemispheres.	Cerebellum.	Cerebral hemispheres.	Cerebellum.
	<i>mg. per 100 gm.</i>	<i>mg. per 100 gm.</i>	<i>per cent</i>	<i>per cent</i>
Rabbit..... 1	113.2	138.0		
2	111.9			
3	116.1			
4	117.5	142.3	1.71	
5	114.2		1.73	
6	116.1			
Average.....	114.8	140.2	1.72	1.86
Cat..... 1	132.3	164.6	1.85	1.93
2	122.9	156.1	1.71	1.83
3	120.3	155.4		
4	126.8	153.6	1.81	
5	115.3	147.3	1.74	
Average.....	123.5	155.4	1.77	1.88
Dog..... 1	118.9	124.9	Whole brain.	
2	120.5	148.0		
3	117.3	124.6		
4	113.2	131.0	2.00	1.96
5	116.1	139.1	1.62	1.67
6	130.9	134.0	1.88	1.91
7	111.4		1.74	
Average.....	118.3	133.6	1.81	1.85
Man.				
Myocarditis..... 1	126.5	192.4	1.78	1.84
Arsenic poisoning.. 2	127.1		1.79	
Stabbing, followed by acute peritonitis..... 3	119.1	187.8	1.77	1.95
Fractured skull.... 4	120.1	171.9	1.88	1.90
5	127.9	168.7		
Pneumonia..... 6	118.6	169.3		
Fractured skull.... 7	119.6	166.2		
Average.....	122.7	176.0	1.80	1.89

one brain upon which to make a determination in the hemispheres separately), the amount of creatine per gm. of N is very constant in the hemispheres. The ratio is not so constant in the cerebellum, and it is indubitably higher than in the hemispheres. The number of determinations on the cerebellum is very much smaller than on the hemispheres, because of the greater difficulty of obtaining sufficient material, and bearing this in mind it is probable that the ratio is a constant for the cerebellum also, but of greater magnitude.

We have also determined the creatine content of the white matter of beef brain in two specimens. These were found to contain 81 and 86 mg. of creatine per 100 gm. of tissue. White matter would thus appear to contain far less creatine than gray. The N content of white matter was found to be 1.625 and 1.715 per cent in the two specimens, respectively, making the relationship of milligrams of creatine to 1 gm. of N as 49.9 and 50.1. Probably the admixture of white matter with the gray may help to account for the difference in creatine content found between the hemisphere and the cerebellum, as although the white matter was dissected out in *gross* it is not possible to effect its complete separation. The cerebellum is known to possess a higher amount of gray matter than the hemispheres.

Autolysis.

The effect of even a short period of autolysis is to lower rapidly the creatine content of the brain and emphasizes the importance of obtaining strictly fresh material for examination.

Material.	Amount in hemispheres.	Amount in cerebellum.
	<i>mg. per 100 gm.</i>	<i>mg. per 100 gm.</i>
Fresh.....	119	169
36 hrs. refrigeration.....	115	158

Another brain received in the laboratory 48 hours after autopsy and showing signs of putrefaction gave: hemispheres, 94 mg.; cerebellum, 139 mg. per 100 gm. It is also of interest to note that the brain of a case of encephalitis lethargica, which was received absolutely fresh, showed a lowered content of creatine—hemi-

spheres, 112 mg.; cerebellum, 158 mg. per 100 gm.—resembling normal brain after a short period of autolysis. The sections of this brain showed degenerative patches throughout the whole area.

TABLE II.

Animal.	Cerebral hemispheres.	Cerebellum.
	<i>mg. creatine per 1 gm. N</i>	<i>mg. creatine per 1 gm. N</i>
Pig.....	68.5	72.7
Cow.....	64.3	72.0
Calf.....	65.6	76.9
“ fetal.....	(82)	
Sheep.....	68.8	80.8
Rabbit.....	66.7	75.4
Cat.....	69.1	82.4
Dog.....	65.3	72.2
Man.....	68.1	93.1

Creatine Content of Lobes.

We examined the creatine content of the different areas of the cerebral hemispheres of man to see if any difference would be detected between the motor areas and the so called silent areas.

Area.	Creatine.	
	Pneumonia.	Encephalitis lethargica.
	<i>mg. per 100 gm.</i>	<i>mg. per 100 gm.</i>
Motor.....	118	113
Frontal.....	119	110
Parietal.....	117	110
Temporal.....	119	114

No difference in creatine content of the different lobes is to be detected, and it is to be noted that in the case of encephalitis lethargica the differing areas are equally affected.

Effect of Fasting and Creatine Feeding.

The influence of fasting upon the creatine content of voluntary muscle is first an increase in the amount of creatine, followed by a diminution. This increase in the creatine content of muscle on fasting has been noted by Myers and Fine (8), and by Mendel and Rose (9), both sets of observers using the rabbit as the experimental animal. We have also used this animal.

The animals were fed a liberal carrot diet for 3 days previous to a 3 day fast. Six rabbits were used for fasting, the six rabbits quoted in Table I acting as controls. The results are shown in

TABLE III.

Fasting.			Creatine feeding.		
Rabbit No.	Creatine.		Rabbit No.	Creatine.	
	Hemispheres.	Cerebellum.		Hemispheres.	Cerebellum.
	<i>mg. per 100 gm.</i>	<i>mg. per 100 gm.</i>		<i>mg. per 100 gm.</i>	<i>mg. per 100 gm.</i>
1	109	128	1	109	140
2	111		2	110	
3	109		3	110	
4	112	140			
5	111				
6	115				
Average.....	111	134	Average....	110	140
Controls.....	115	140	Controls....	108	129

Creatine in muscle fasting 584 mg. per 100 mg. Average of 12 analyses.
 " " " control 570 " " 100 " " " 12 "

Table III, and although compared with the controls, the brains (both hemispheres and cerebellum) of the fasted animals contain a slightly lower content of creatine, yet we would not say that fasting diminishes the creatine content. The results are within experimental variation. For the determination of the muscle creatine a cross-section of the thigh of the rabbit was used for analysis, and here the results confirm those of previous observers. There is a slight increase in muscle creatine, which would appear to be just outside the range of experimental variation. Certainly,

comparing the effect of fasting upon the creatine content of brain and muscle, the results do not go in the same direction.

The effect of creatine feeding is well known to increase the percentage of creatine in muscle. In studying the effect of creatine feeding upon brain, we again used rabbits as the experimental animals. Three rabbits fed on a liberal carrot diet were given 0.125 gm. of creatine hydrate in 15 cc. of water by stomach tube twice daily. This is a very high dosage, being 125 mg. of creatine per kilo of body weight. This was continued for 7 days. The effect of this administration of creatine on the muscles will be reported in detail later. It would appear, however, to have no effect upon the creatine content of the brain. The hemispheres remain absolutely constant in the amount of creatine compared with the hemispheres of rabbits kept under identical conditions. The creatine content of the cerebellum of the creatine-fed rabbits is higher than that of the controls, but the increase is not beyond that amount which we have noted in other rabbits not so fed.

SUMMARY.

1. The creatine content of brain expressed as milligrams per 100 gm. is a constant varying for each species.
2. The cerebellum has always a higher content of creatine than the hemispheres.
3. The lobes of the hemispheres possess the same creatine content.
4. Autolysis rapidly lowers the creatine content of brain.
5. The conditions of fasting and of creatine feeding do not affect the creatine content of brain.
6. Expressed as milligrams of creatine per gm. of N the creatine content of the hemispheres is a constant independent of the species. This is probably also true of the cerebellum although the constant has a higher numerical value.

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BLOOD CHANGES AND CLINICAL SYMPTOMS FOLLOWING ORAL ADMINISTRATION OF PHOSPHATES.

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It was shown by Binger (1) that intravenous injection of *o*-phosphates to the amount of 150 mg. of phosphorus per kilo of body weight produces a drop of the serum calcium from the normal level of 10 mg. per 100 cc. to approximately 6 mg. This drop is accompanied by symptoms of tetany, provided alkaline or neutral phosphate solutions are employed. With solutions more acid than pH 6 no symptoms occur, although the same drop in serum calcium is observed. Binger concluded that the tetanic condition caused by the injection of phosphates is intimately associated with a decrease in serum calcium, but not dependent on this alone, since a drop in calcium may occur without the appearance of tetany. Greenwald (2) maintains that the toxic symptoms following injection of sodium salts are due to "sodium poisoning" and independent of the anion, and the drop in calcium observed by Binger (1) is due to dilution of the blood by the large amount of fluid introduced with the phosphates. Tisdall (3), however, produced the same drop in calcium by using stronger solutions, whereas the other ions remained constant, showing that no appreciable dilution had taken place.

There are other observations indicating a causal relation of low blood calcium to high phosphates. In nephritis with kidney insufficiency, presumably in cases with acidosis and impending or manifest uremia, this change is one of the characteristics of the blood as demonstrated by Marriott and Howland in 1916 (4). In parathyroid insufficiency the characteristic drop in blood calcium seems to be always accompanied by an increase in the phosphates, as first found by Greenwald (5) and confirmed by

others (6), although this increase sometimes is not very marked in the more chronic conditions of tetany.

Jeppsson and Klercker (7) produced increased electrical and mechanical irritability in dogs and infants by giving alkaline phosphates, presumably the potassium salt, by mouth. In one child suffering from diabetes insipidus they observed carpopedal spasm and in two dogs they saw light spasms of the hind legs following the administration of alkaline potassium phosphate to the amount of 0.20 gm. of phosphorus per kilo of body weight. The interpretation of their results with alkaline salts is difficult in light of the question of the supposed relation between alkalosis and tetany. Elias and Kornfeld (8) saw an aggravation of the tetanic condition after the administration of phosphates. Elias (9) had also produced increased electrical irritability by the administration of different acids, including acid phosphates.

The object of the present experiments was to study the effect of oral administration of phosphates on the blood and the relation of any symptoms that might occur to the changes produced in the blood. Because of the possible causal relationship of certain kinds of tetany to alkalosis and the fact that acid administration may relieve tetanic symptoms, particular care was taken to give the phosphates in a solution of the same pH as the blood. Later, also, the effects of alkaline and acid phosphates were studied.

EXPERIMENTAL.

The experiments were arranged in two series: in the first series small doses of phosphates were given over a long period, in the second series large doses were given over a short time. Only sodium salts were used, as potassium is toxic in large doses and the interpretation of the symptoms might be difficult. The inorganic bases, chlorine, inorganic phosphorus, carbon dioxide content, and pH were determined and also the total serum protein, as changes in this constituent of the blood may cause changes in the blood calcium (Salvesen and Linder (10)). In two of the experiments the blood sugar was determined. The phosphate solutions were given by stomach tube.

Methods.

The serum constituents were determined by the following methods: Inorganic bases by the methods of Kramer and Tisdall (11); phosphates by the method of Tisdall (12); chlorides by the new method of Van Slyke (13), using 1 cc. of serum for each determination; CO₂ content by the Van Slyke constant volume apparatus (14); and pH electrometrically, using as a standard of reference 0.10 N HCl and assuming as its pH at 38°, 1.090, and in some instances colorimetrically as well by the Cullen method (15). Total protein of serum was determined by the method of Howe (16). The blood was always collected under oil without stasis, centrifuged at once, and the serum kept over mercury.

1. Administration of Small Doses of Phosphates over a Long Period.

Three different solutions of phosphates were prepared. The phosphate solution *isohydronic* with blood contained 2.6 gm. of NaH₂PO₄·H₂O + 30.7 gm. of Na₂HPO₄·12H₂O per liter and was adjusted to a pH of 7.4 by the addition of 10 per cent HCl or NaOH. The *alkaline* phosphate solution contained 37.5 gm. of Na₂HPO₄·12H₂O per liter and the *acid* solution contained 14.4 gm. of NaH₂PO₄·H₂O per liter. The content of *phosphorus* was the same in all three solutions; namely, 3.25 gm. per liter. Blood for normals was drawn in the morning before meals. In the first two experiments with neutral phosphates 300 cc. of the phosphate solution (= 0.975 gm. of phosphorus) were given daily for a week; then the dose was increased to 450 cc. (150 cc. three times a day = 1.46 gm. of phosphorus) for 4 days. In the experiments with alkaline and acid phosphates 450 cc. (150 cc. three times a day = 1.46 gm. of phosphorus) were given from the start. Blood was collected again at the close of the experimental period; it was taken at 10 a.m., 1 hour after one dose of the phosphates (150 cc.) was given.

Results.—The experiments are recorded in Tables I to III, in which all the values of the inorganic constituents are given in millimols per liter of serum. It will be seen that the administration of *neutral* phosphates produced a considerable increase in the phosphates of serum accompanied by a decided drop in

TABLE I.
Effect of Oral Administration of Neutral Phosphate on the Composition of Serum.
 Phosphate solution contained 2.6 gm. of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 30.7 gm. of $\text{NaHPO}_4 \cdot 12\text{H}_2\text{O}$ per liter. pH 7.4.

Weight. kg.	Date. 1935	Per liter of serum.										Remarks.
		N	K	Ca	Mg	Inorganic P.	Cl	CO_2 content.	Sum of Columns 4 to 10.	pH	Protein.	
		mm	mm	mm	mm	mm	mm	mm	mm		gm.	
14.0	Dec. 1	144.3		2.85	0.62	1.7	109.5	24.15		7.36	54.7	From Dec. 1 to 10, 0.975 gm. P a day. " " 10 " 14, 1.462 " " " " " 14, no phosphates.
	" 10	144.3		2.65	0.54	2.3	103.9	25.58		7.39	55.2	
	" 14	142.0		2.37	0.49	3.0	102.4	24.9		7.39	57.9	
	" 18			2.60		1.58						
15.0	" 1	144.8	5.5	2.89	0.63	1.93	110.5	24.13	290.38	7.42	57.5	From Dec. 1 to 10, 0.975 gm. P a day. " " 10 " 14, 1.462 " " " " " 14, no phosphates.
	" 10	145.4	5.3	2.71	0.53	1.97	108.4	25.62	289.93	7.45	55.1	
	" 14	146.1	4.9	2.39	0.48	3.10	104.2	26.08	287.25	7.41	57.8	
	" 18			2.80		1.10						

TABLE II.
Effect of Oral Administration of Alkaline Phosphate on the Composition of Serum.
 Phosphate solution contained 37.5 gm. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ per liter.

LOG NO.	Weight. kg.	Date.	Per liter of serum.										Remarks.
			Na	K	Ca	Mg	Inorganic P.	Cl	CO_2 content.	Sum of Columns 4 to 10.	pH	Protein. gm.	
3	20.0	1934 Jan. 17	mm 140.4	mm 6.2	mm 2.38	mm 0.53	mm 1.64	mm 106.2	mm 19.6	mm 277.2	mm 7.30	gm. 64.4	From Jan. 17 to 23, 1.462 gm. P a day.
		" 23	mm 159.1	mm 5.8	mm 2.81	mm 0.57	mm 1.84	mm 102.8	mm 28.53	mm 301.4	mm 7.40	gm. 62.2	
5	14.8	" 17	mm 148.1	mm 5.9	mm 2.92	mm 0.59	mm 1.40	mm 106.0	mm 22.7	mm 287.6	mm 7.39	gm. 57.9	From Jan. 17 to 23, 1.462 gm. P a day.
		" 23	mm 166.1	mm 5.1	mm 2.80	mm 0.57	mm 2.10	mm 104.7	mm 26.8	mm 308.1	mm 7.42	gm. 55.6	

TABLE III.
Effect of Oral Administration of Acid Phosphate on the Composition of Serum.
 Phosphate solution contained 14.4 gm. of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ per liter.

Weight. kg.	Date.	Per liter of serum.										Remarks.
		Na mm	K mm	Ca mm	Mg mm	Inorganic P. mm	Cl mm	CO_2 content. mm	Sum of Columns 4 to 10. mm	pH	Protein. gm.	
19.6	Feb. 8 1934	147.0	5.7	2.96	0.64	1.63	107.8	24.17	289.4	7.33	68.5	From Feb. 8 to 14, 1.462 gm. P a day.
"	" 14	152.6	6.1	2.96	0.59	2.32	108.5	23.70	296.7	7.37	66.7	
14.8	" 8	149.1	5.7	3.03	0.62	1.55	108.0	24.14	292.4	7.36	63.0	From Feb. 8 to 14, 1.462 gm. P a day.
"	" 14	151.2	4.3	2.79	0.58	3.30	104.3	25.34	291.81	7.35	61.8	

the calcium (of about 17 per cent). There was also a drop in the magnesium and a considerable decrease in the chlorides. The blood calcium, determined 4 days after the administration of phosphates was discontinued, showed a normal value for one dog and a subnormal value for the other. *Alkaline* phosphate produced no change in serum calcium¹ and only a slight increase in the phosphate content; sodium and CO₂ content increased considerably, while there was a slight decrease in chlorides. The alkalinity of the blood increased somewhat in both of the dogs. *Acid* phosphates produced no appreciable change in the calcium content, but a marked increase in the phosphate content. In none of the experiments of this series were there any clinical symptoms.

2. Administration of Large Doses of Phosphates over a Short Period.

The dogs used were the same that had been employed in the foregoing experiment, allowing a certain time to elapse, so that the effect of the previous experiments had disappeared. The concentration of the phosphate solution was trebled, so they contained 9.75 gm. of phosphorus per liter. Blood for normals was either taken in the morning before the first dose was given or at 5 p.m. the preceding day. On the day of the experiment 150 cc. of the phosphate solution (corresponding to 1.462 gm. of phosphorus) were given three or four times at about 2 hour intervals, and in a few experiments also a fifth dose of 100 cc. (0.975 gm. of P). Only in the first experiment were the doses given less frequently and over 2 days. The dogs had free access to water.

Results.—The most violent clinical symptoms were observed in all the experiments, no matter whether neutral (pH 7.4), alkaline, or acid phosphates were employed. After the third or fourth dose the dogs became quiet, lost their spirit, seemed to be uncomfortable, and all had a peculiar symptom, which often was seen in latent tetanic dogs by one of us (6) and which seemed to be

¹ Dr. B. Kramer and his associates have noticed a decrease in the blood calcium of rachitic children to whom alkaline sodium phosphate was given (personal communication).

	Mar. 3, 7.30 p.m.	149.4	6.5	1.89	0.49	2.48	108.2	25.13	294.0	7.28	*59.7	At 6.30 p.m., tetany. " 7.55 " intravenously 1 gm. CaCl ₂ . No symptoms.
	" 3, 8.15 "		4.68	0.53	2.71							
5 15.4	" 4	147.9	6.2	3.12	0.64	1.71	111.5	22.53	293.73	7.38	62.00	Mar. 5. 1.462 gm. P administered at 9 a.m., 11.15 a.m., 1.15 p.m., and 3.15 p.m. and 0.975 gm. at 4.15 p.m.; 6.823 gm. in all.
	" 5, 6.30 p.m.	149.7	6.3	1.63	0.46	3.60	110.0	26.05	298.7	7.38	60.40	Violent tetany at 7.45 p.m., 0.7 gm. CaCl ₂ intravenously.
	" 5, 8.45 "			2.79	0.49	2.42						No symptoms.

* Colorimetric.

a sure sign of approaching tetany; the dogs started to scratch their eyes and nose or rubbed the face against the wall or the floor, as if something was itching. Also an increased salivation was noticed at this stage. All the typical symptoms of the condition which is called tetany now gradually developed: rigidity of the legs, dragging of the toes, twitchings of different muscle groups, particularly of shoulders, hips, and head; sometimes a marked trismus was observed. Finally the dogs could not move, lay down with extension spasm of all four legs, twitchings in different muscle groups, salivation, and a most marked dyspnea; they tried to drink, when offered water, but could not. When these symptoms were produced, blood was taken for analysis and 0.5 to 1.0 gm. of calcium chloride in 10 per cent solution was injected into the heel vein. The effect of the calcium administration was immediate: the convulsions usually stopped during the injection; the dyspnea first increased for a few moments and then subsided; the dogs rose, drank large amounts of water, and in the following 15 minutes became completely normal to all appearances, were playful, and could run up and down the corridor at full speed without displaying any symptoms. Blood was taken again for analysis when this effect was produced. One dog was not given calcium and recovered spontaneously (Dog 3, Table IV). Phosphates were given again the next day, produced twitchings, and the dog was found dead in the evening. Another dog, which was prepared for demonstration for the staff of the hospital and had been given alkaline phosphates during the night, showed the characteristic symptoms of tetany in the morning, but the dog seemed then to pass into a state of paralysis. It was lying on the floor with closed eyes, panting, and seemed to be completely limp; it fell down in a heap when raised on its legs. The injection of calcium chloride produced violent convulsions and before a second dose could be given the dog died during an attack of epileptiform convulsions with opisthotonos, trismus, and spasm of the diaphragm. No blood was taken from this dog during this experiment; the dog had been used for similar experiments before and had showed the ordinary reaction. In some of the dogs the phosphate administration caused diarrhea.

The experiments are recorded in Tables IV and V. It will be seen from the tables that the changes produced in the blood

Effect of Oral Administration of Large Doses of Alkaline and Acid Phosphates on the Composition of Serum.

Alkaline phosphate contained 112.5 gm. of $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ per liter.

Acid phosphate contained 43.3 gm. of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ per liter.

Dog No.	Weight. kg.	Date.	Per liter of serum.											Remarks.
			Na	K	Ca	Mg	Inorganic P.	Cl	CO ₂ content.	Sum of Columns 4 to 10.	pH	Protein.	Blood sugar.	
		1924	mm	mm	mm	mm	mm	mm	mm	mm	mm	mm	gm.	gm.
4	13.2	Mar. 10, 7.45 p.m.	149.1	4.82	800.622	15	105.8	26.3	291.5	7.38	54.0	0.91	Alkaline phosphate. Mar. 11. 1.462 gm. P administered at 9 a.m., 11 a.m., and 1 p.m. and 0.975 gm. at 3 p.m.; 5.361 gm. in all. Violent tetany. At 5 p.m., 0.5 gm. CaCl ₂ intravenously. No symptoms.	
		" 11, 4.45 "	149.3	5.61	580.473	90	105.4	28.48	294.7	7.27	56.0	0.99		
		" 11, 5.10 "			4.620	49	4.55							
		" 13, 4.30 "	142.3	6.73	060.651	8	107.0	21.8	283.31	7.36	60.7		Acid phosphate. Mar. 14. 1.462 gm. P administered at 9 a.m., 11 a.m., 1 p.m., and 3 p.m. and 0.975 gm. at 4 p.m.; 6.823 gm. in all. Marked tetany. At 6.20 p.m., 0.7 gm. CaCl ₂ intravenously. Normal.	
5	15.6	" 14, 5.25 "	145.6	5.61	650.463	10	105.4	22.6	284.4	7.38	61.3			
		" 14, 6.35 "			3.840	42	3.65							

picture are uniform and independent of the pH of the phosphate solution. The characteristic change is a rise in the phosphates and a drop in the blood calcium. The phosphates increased to a minimum of 2.5 mm and a maximum of 3.9 mm per liter. The serum calcium decreased from a normal average of 2.96 mm to a minimum of 1.58 mm and a maximum of 1.89 mm per liter (corresponding to 6.3 and 7.5 mg. per 100 cc.). There was also a drop

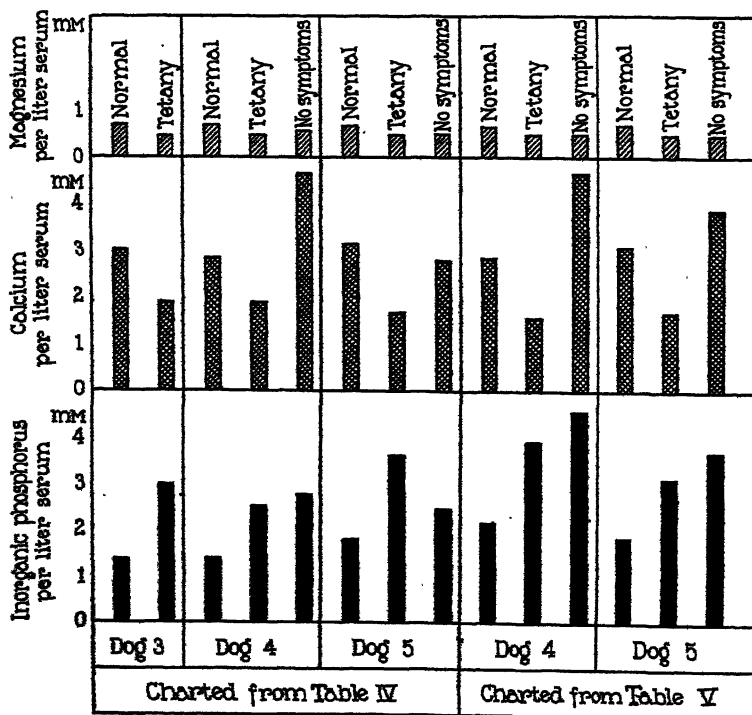


FIG. 1. Relation between phosphates, calcium, and magnesium per liter of serum before the administration of phosphates, during tetany, and after the administration of calcium chloride.

in the magnesium in all the experiments. In some of the experiments there was a rise in the sodium content, especially in the first experiment, where the increase was 7 mm. The pH kept constant in most of the experiments; in others, small changes occurred, both to the alkaline and the acid side. There was no marked drop in the chlorides as in the first series of experiments.

The blood sugar decreased a little in one of the experiments in which the determination was done. The serum proteins did not change appreciably.

Blood taken after the dogs became normal again by calcium administration showed calcium values above the normal and a further increase in the phosphates except in one experiment (Dog 5, Table IV) in which the calcium did not rise above the normal value and the phosphates were lower after the calcium injection.

The relationships between phosphates, calcium, and magnesium of serum are seen from Fig. 1.

DISCUSSION.

The clinical symptoms observed after oral administration of phosphates might be due to: (1) salt action and change in the osmotic condition of the body, (2) specific toxic action of the phosphate ion, and (3) decrease in blood calcium.

1. Large amounts of a hypertonic salt solution were introduced into the intestinal tract. The increase produced in the molecular concentration of inorganic ions of the blood was, however, negligible (see Column 11 of Tables IV and V). It was less marked than in some of the experiments of the first series, in which no clinical symptoms were observed (Table II). The symptoms, therefore, cannot be due to changes in the osmotic conditions of the blood.

2. The increase in the phosphate concentration of serum was very irregular and had no relation to the symptoms produced, as the phosphates rose to higher figures in some of the experiments of the first series, in which no symptoms were produced, than those observed in some of the experiments of the second series.

3. The decrease in serum calcium has a definite relation to the clinical symptoms. In the first series the calcium dropped only a trifle or not at all, and no symptoms occurred. In all the experiments of the second series, a low blood calcium was found. The parallelism between the blood changes and symptoms found in the present experiments and those found in parathyroid insufficiency is striking. To those of us who had had previous experiences with parathyroidectomized dogs, the symptoms produced by oral phosphate administration were identical with

those of parathyroid insufficiency (6, 17). And it has been shown lately by Salvesen (6) that all the symptoms of parathyroidectomy are due to calcium deficiency, as first maintained by MacCallum and Voegtlin (18). Calcium administration promptly relieves the symptoms of both conditions. The tetanic symptoms develop in parathyroidectomized dogs when the blood calcium has dropped to approximately 7 mg. per 100 cc. of serum (1.75 mm. per liter) (Hastings and Murray (17)). In three of the experiments reported in this paper the blood calcium was below 7 mg. (1.75 mm.) when tetany occurred; in the remaining two it was 7.5 mg.; in these two experiments there was also an increase in the sodium content of serum. This is probably of importance, as it is the sodium: calcium ratio that determines the irritability of muscles and nerves according to Loeb (19). The decrease in magnesium, caused by the phosphate administration is probably also of importance.

It seems justifiable to conclude that the symptoms observed after phosphate administration are due to the calcium deficiency produced in the blood. There remains to be explained the mechanism of the calcium decrease. Starkenstein (20) and Binger (1) suggested that the injected phosphates precipitate the calcium. The concentration of phosphates in serum, when the calcium decrease was established, averaged 18.5 mg. of phosphorus per 100 cc. (6 mm per liter) in Tisdall's experiments (3). In our experiments the maximum phosphate increase was 3.6 mm (11.2 mg.) and might be as low as 2.5 mm (7.75 mg.) when tetany occurred. The concentration of phosphates in the blood had no relation to the drop in calcium, as in the first series of experiments the phosphates rose to a similar height without changing the calcium appreciably (Table III). This observation does not seem to favor the view that the mere increase in the phosphate concentration of serum, caused by oral administration of phosphates, forces the calcium down by precipitation; if that had been the case, one would expect the calcium to be lowest in serum, which showed the highest phosphates.

Another possibility is that the oral administration of phosphates causes an increased excretion of calcium (and magnesium), probably in the way that phosphates are constantly taken up as sodium salts and partly excreted as calcium and magnesium

salts. Jeppsson and Klercker (7) observed an increased excretion of calcium and partly also magnesium through the gut during oral administration of phosphates. They regard it as excluded, however, that the spasmophilic symptoms observed in their experiments are only due to a depletion in the calcium content of the organism.

Whether the results of the present experiments have any bearing upon the question of how the low blood calcium in parathyroid insufficiency is produced, it is too early to say, but they serve as a further proof for the view that the symptoms of parathyroid insufficiency are due to the low blood calcium. It seems also to be certain from these experiments and from those of Binger (1) that the phosphate retention in uremia is the cause of the excessive drop in blood calcium, which cannot be accounted for by the decrease in plasma protein (10).

SUMMARY.

The changes in the blood and the clinical symptoms of dogs to whom phosphate solutions were given orally have been studied. Small doses of phosphates given over a long period (1.46 gm. of phosphorus a day, corresponding to 0.07 to 0.10 gm. per kilo of body weight) did not produce any clinical symptoms. If alkaline phosphates were used, no change was observed in the calcium and inorganic phosphorus content of the blood. If acid phosphates were used, there was a marked increase in the serum phosphates, but no change in the calcium. Solutions of the same pH as that of blood produced a marked increase in phosphates and a decrease in calcium of 0.5 mm.

Larger doses of phosphates (0.40 to 0.70 gm. of phosphorus per kilo of body weight), administered over a period of 1 (or 2) days, produced violent symptoms, which had the characteristics of the so called *tetany* and which were independent of the pH of the solution administered. The serum phosphates increased to values varying between 2.5 to 3.9mm; the calcium decreased from values which ranged between 1.89 to 1.58 mm. There was also a decrease in the magnesium content of serum and in one instance a decrease in the blood sugar. The injection of calcium chloride checked all the symptoms and produced an increase in the serum calcium.

CONCLUSION.

The clinical symptoms (tetany), produced by the oral administration of phosphates, are due to a reduction of the blood calcium.

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THE EFFECT OF THE ADMINISTRATION OF CALCIUM SALTS ON THE INORGANIC COMPOSITION OF THE BLOOD.

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Calcium chloride, which is the most commonly used calcium salt in the treatment of tetany, has lately been reported to cause a marked acidosis, when administered orally. Gamble, Ross, and Tisdall (1) concluded from their experiments on two infants that ingested calcium chloride behaves as an acid substance, due to much greater absorption of the chlorine than of the calcium. In the urine they observed a considerable increase in the acid excretion and in the blood an increase in the chloride concentration, accompanied by an equimolecular decrease in the bicarbonate, while the total base concentration was unaltered. They did not determine the pH of the blood, but Gamble and Ross assume that calcium chloride has the same effect as hydrochloric acid, which lowered the pH in an infant to whom it was given orally (2). At the same time the observations of Atchley, Loeb, and Benedict (3), who studied the calcium chloride diuresis, were published. As the result of calcium chloride ingestion, they found in a diabetic individual with edema an approximately molecular replacement of bicarbonate by chloride in the blood with little change in the total base, whereas in a normal person both the sodium and the carbon dioxide concentrations were decreased. It was demonstrated as early as 1915 by Wilson, Stearns, and Janney (4), that the ingestion of hydrochloric acid relieves the tetanic symptoms of parathyroidectomized dogs, and lately by Sheer and Jahr (5) that hydrochloric acid, added to milk, relieves the symptoms of infantile tetany. Also ammonium chloride, the ingestion of which is followed by the typical signs of

an acidosis (Haldane (6)), is almost as efficient as calcium chloride in relieving tetany, according to Freudenberg and György (7) and Gamble and Ross (2). These observations seem to raise doubt as to the manner in which calcium salts relieve tetanic symptoms. It has previously been thought that calcium acts by raising the low blood calcium, which is regarded as the cause of infantile and parathyroid tetany. From the above results it appears that relief of tetany by orally administered calcium salts may be due not to the calcium, but to the anion of the salt, which is absorbed as an acid. Such an explanation would be in harmony with the hypothesis of Wilson, Stearns, and Thurlow (8) that tetany is caused by alkalosis.

The present experiments were undertaken in order to study the effect of oral and intravenous administration of calcium salts on the acid-base equilibrium and the inorganic constituents of the blood. An attempt was also made to trace the fate of intravenously introduced calcium chloride.

EXPERIMENTAL.

The experiments were performed on normal dogs and on two nephritic patients, to whom calcium salts were administered as therapeutic agents to produce diuresis.

Methods.

The inorganic bases of serum were determined by the methods of Kramer and Tisdall (9); phosphates by the method of Tisdall (10); chlorides by the new method of Van Slyke (11), using 1 cc. for each determination; carbon dioxide content by the Van Slyke constant volume apparatus (12); and pH electrometrically in the serum of dogs, using 0.10 N hydrochloric acid as a standard of reference and assuming as its pH at 38°, 1.090. In human serum pH was determined colorimetrically by the Cullen method (13). Plasma protein was determined by the method of Howe (14). Blood was collected under oil without stasis, centrifuged at once, and the serum kept over mercury.

TABLE I.
Effect of Oral Administration of Calcium Chloride (Dogs 1 and 2) and Calcium Lactate (Dog 3) on the Composition of Serum.

Dog No.	Date.	Weight. kg.	Per liter of serum.										Per liter of plasma.		Remarks.
			Na	K	Ca	Mg	Inor- ganic P.	Cl	CO ₂ con- tent.	BHCO ₃	CO ₂ ten sion.	pH	Pro- tein.	Non- protein N.	
			mm	mm	mm	mm	mm	mm	mm	mm	mm	mm	mm.	gm.	
1	1923 Oct. 17 " 20	12.4 12.5	147.8 139.1	5.4 6.5	3.10 2.98	0.66 0.49	2.58 1.94	106.1 113.5	24.08 15.43	23.05 14.40	32.7 33.5	7.47 7.27	52.3 58.4	0.42 0.28	Oct. 18. 15 gm. CaCl ₂ . " 19. 20 "
2	" 25 " 27	15.8 15.0	154.7 144.3	4.9 6.1	2.75 3.37	0.57 0.53	1.22 1.55	105.4 118.9	26.25 12.18	25.02 11.09	40.1 35.6	7.43 7.13	64.0 68.8	0.23 0.19	" 25. 15 " " 26. 18.7 " " 27. 7.5 " at 9.00 a.m.; blood taken at 10.45 a.m.
3	Nov. 3	20.0	145.6	5.7	3.01	Lost.	1.40	108.9	24.54	23.37	38.2	7.42	63.6	0.52	Nov. 4, 5, and 6. 20 gm. Ca lactate a day.
" 7			152.1	6.0	3.00	"	1.03	109.3	25.70	24.58	36.7	7.46	60.4	0.42	Nov. 7. 10 gm. Ca lactate at 8.55 a.m.; blood drawn at 11.30 a.m.

1. Oral Administration of Calcium Chloride and Calcium Lactate.

Dogs.—The administration of the calcium salts by stomach tube to two dogs was carried out as follows: To one of the dogs 15 to 20 gm. of calcium chloride in 5 per cent solution were administered on each of 2 days and blood was taken on the morning of the 3rd day; in the other dog the same amount of calcium chloride was given for 2 days and on the 3rd morning 7.5 gm. were given at 9 a.m. and blood was drawn at 10.45 a.m. A third dog was given 20 gm. of calcium lactate in 5 per cent solution (by stomach tube) for 3 days and on the 4th day 10 gm. were given in the morning and blood was taken 2½ hours after. The dogs were kept on a constant diet of bread and soup and had free access to water.

Results.—The dogs to which calcium chloride was administered became depressed and lost appetite; they had no apparent dyspnea. Calcium lactate had no clinical effect. The experiments are recorded in Table I, in which the results are expressed in millimols per liter of serum. It will be seen that calcium chloride produced characteristic changes in the composition of the blood; chlorides increased and bicarbonate decreased almost equimolecularly, while the sodium decreased 8.7 millimols in one case and 10.4 in the other, leaving the blood markedly more acid on account of the actual loss of base as well as the increase in fixed acid; the pH dropped in the first animal from 7.47 to 7.27, in the second from 7.43 to 7.13. There was an increase of 20 per cent in the blood calcium of the latter dog. There was a slight rise in potassium and an increase in the concentration of plasma protein. Calcium lactate produced a small increase in the pH and the sodium content. The CO₂ tension, calculated from the CO₂ content and the pH, was not significantly changed.

Human Subjects.—The effect of oral administration of calcium salts was studied in two young men, one suffering from acute glomerular nephritis, with extensive edema, while the other was a case of chronic nephrosis with some edema of legs and lumbar region. Neither had retention of urea. The patient with glomerular nephritis was given 10 gm. of calcium lactate daily for 9 days and then 13.5 gm. of calcium chloride (in two doses of 6.75 gm.) for 4 days. The other patient was given 15 gm. of

TABLE II.
Effect of Oral Administration of Calcium Lactate and Calcium Chloride on the Composition of Serum in a Case of Acute Glomerulonephritis, and of Calcium Chloride in a Case of Chronic Nephrosis.

Name. Sex. Age. Diagnosis.	Date.	Weight. kg.	Per liter of serum.										Per liter of plasma.				Remarks.	
			Na	K	Ca	Mg	Inor- ganic P.	Cl	CO ₂ con- tent.	HCO ₃	CO ₂ ten- sion.	pH	Total pro- tein.	Albu- min.	Glob- ulin.	Albumin Globulin		Non- pro- tein N.
J. M. Male. 24	1924 Jan. 22	68.2	152.0	4.9	1.99	0.78	1.23	100.0	29.2	27.68	49.7	7.38	39.2	13.2	26.0	0.50	0.34	From Jan. 25 to Feb. 2, 10 gm. Ca lactate a day.
Acute glo- merulone- phritis.	" 30	68.7	141.0	5.9	1.98	0.74	1.43	91.7	29.6	27.95	53.8	7.35	41.0	13.8	27.2	0.51	0.33	From Feb. 3 to 6, 13.5 gm. CaCl ₂ a day.
	Feb. 7	68.5	131.5	7.1	1.96	0.70	1.64	108.4	18.5	16.87	52.5	7.14	36.5	12.3	24.2	0.51	0.41	
B. B. Male. 27	Apr. 9	57.0	139.3	4.7	2.03	0.74	1.55	98.9	33.3	31.80	65.3	7.32	34.2	11.3	22.9	0.49	0.34	From Apr. 9 to 14, 15 gm. of CaCl ₂ a day.
Chronic nephrosis.	" 15	56.3	133.6	4.6	2.05	0.50	1.93	105.6	23.4	21.94	47.6	7.30	41.2	11.4	29.8	0.38	0.14	

calcium chloride (in two doses of 7.5 gm.) a day for 6 days. They were both on a constant, chloride-free diet and on a constant fluid intake.

Results.—In both of the patients the calcium chloride administration caused some abdominal discomfort. In neither of them was there any clinical sign of acidosis, except that the patient with nephritis complained of breathing being "heavy." There was practically no effect on the urinary output and Table II shows that the weight remained almost the same as before the administration. Both patients had a low plasma protein and a corresponding low plasma calcium (15).

Calcium lactate produced no change in the blood, except an equimolecular drop in the sodium and chloride content; this may be the effect of the chloride-free diet, which was given the patient from the day before calcium administration was started. Calcium chloride produced the same qualitative changes in the inorganic constituents as in dogs. In both of the cases there was an actual loss of base; in the first case sodium dropped 9.5 mm and the bicarbonate 11 mm, while the chlorine increased 16.7 mm; there was accordingly an increase of acid over base in the blood at the end of the experiment, which accounts for the low pH of 7.14. The CO_2 tension remained constant. In the second case the sodium dropped only 5.7 mm while the bicarbonate decreased 10 mm. As the chlorine increased 6.7 mm, the relationship between acid and base remained constant and there was no change in the pH of the blood. The CO_2 tension, which was abnormally high at the beginning of the experiment, was much reduced. In none of the cases did the calcium administration raise the blood calcium.

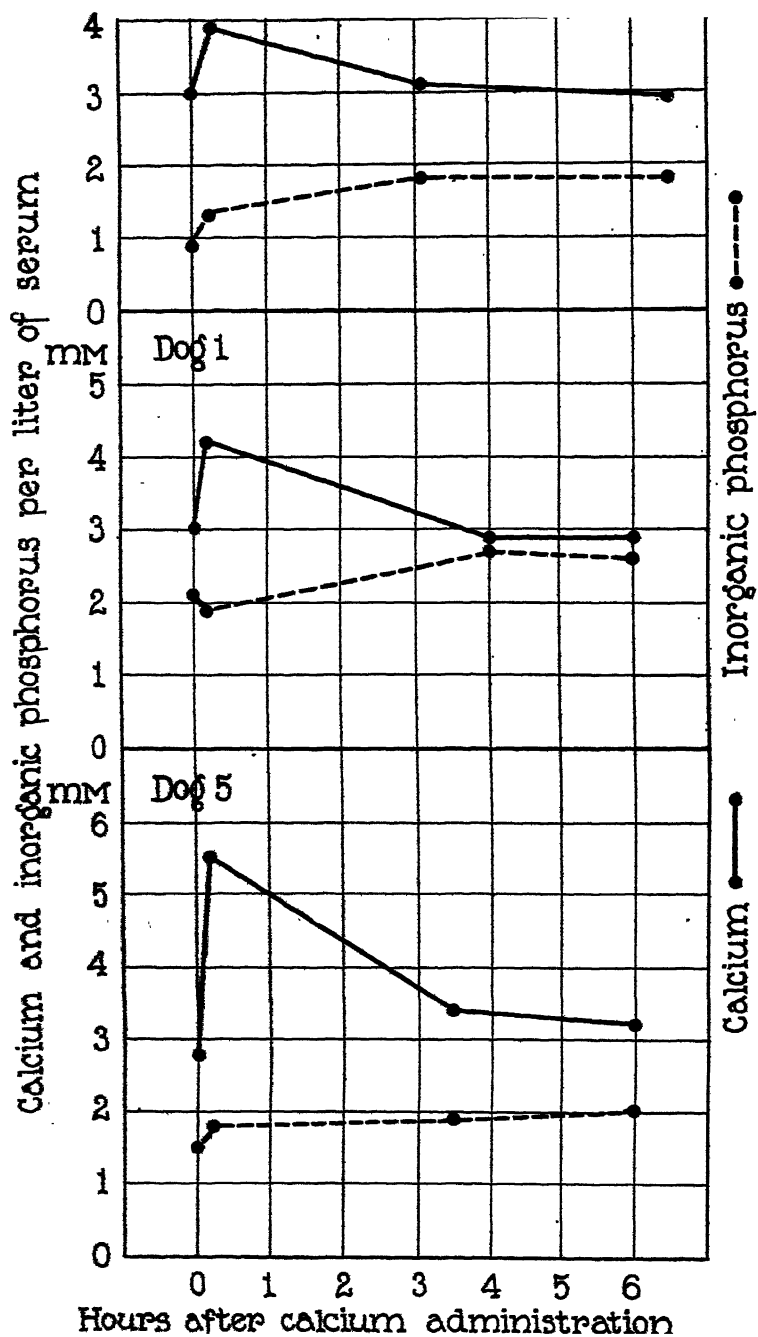
2. Intravenous Administration of Calcium Chloride.

A measured amount of calcium chloride was injected intravenously into three dogs and blood was taken at intervals after the injection. The calcium excretion in urine and feces was studied in two of the dogs as follows: The dogs were given a constant diet of minced meat for 5 days before and for 3 days after the injection. 3 days before the injection the dogs were given high enemata until the colon was completely rinsed out and the

TABLE III.
Effect of Intravenous Injection of Calcium Chloride on the Composition of Serum.

Dog No.	Weight. kg.	Date.	Per liter of serum.								Remarks.	
			Na mm	K mm	Ca mm	Mg mm	Inorganic P. mm	Cl mm	CO ₂ content. mm	pH		
3	19.8	1923-24										
		Nov. 16.	148.2	6.7	3.03	0.72	0.90	107.3	24.9	7.41	10.35 a.m. Injected 0.5 gm. CaCl ₂ in 2.5 per cent solu- tion.	
		10.15 a.m.	152.2	6.9	3.90	0.64	1.29	109.4	25.1	7.38		
		10.50 "										
1.45 p.m.		7.1	3.10	0.52	1.78	105.8	27.6	7.41				
1	12.5	5.20 "	154.3		2.92	0.57	1.78	106.7	27.3	7.44		
		Nov. 23.	145.1	*	2.98	0.66	2.13	104.7	23.6	7.42	10.40 a.m. Injected 0.75 gm. CaCl ₂ in 2.5 per cent solu- tion.	
		10.30 a.m.	143.9	*	4.17	0.54	1.90	105.8	23.2	7.39		
		11.00 "										
2.30 p.m.	147.0	*	2.90	0.59	2.68	106.0	24.5	7.46				
5	14.8	4.45 "	143.9	*	2.92	0.65	2.58	106.9	25.2	7.42		
		Feb. 1.									10.30 a.m. Injected 1.10 gm. CaCl ₂ in 10 per cent solu- tion.	
		10.00 a.m.	147.8	5.2	2.85	0.64	1.50	104.2	25.6	7.42		
		10.50 "	151.2	5.6	5.50	0.62	1.81	103.8	24.3	7.39		
2.00 p.m.	152.4	6.2	3.42	Lost.	1.93	104.9	23.9	7.37				
		4.20 "	150.6	5.7	3.22	0.64	2.00	105.4	23.8	7.37		

* Reagent precipitated by serum.



solution returned colorless.¹ The dogs were then placed in metabolism cages and the urine was collected for the following 3 days. On the 3rd day, which was the day of the calcium administration, the colon was rinsed out again and the feces with the washings were collected. The dogs had had no defecation during these 3 days and the amount of feces obtained represented the feces produced during the 3 day period. Then the calcium was injected and urine collected for the following 3 days. The feces were obtained at the end of the 3 days in the same manner as before, by thorough rinsing out of the colon. The amount of

TABLE IV.

Calcium Secretion in Urine and Feces in a 3 Day Period Before and After the Injection of Calcium Chloride.

Dog No.	Amount of Ca injected.	Period.	Ca in urine.	Ca in feces.	Total.	Increase in excretion after injection.
	gm.		gm.	gm.	gm.	
1	0.271	Before injection.	0.018	0.123	0.141	-0.049
		After "	0.010	0.082	0.092	
5	0.398	Before injection.	0.094	0.660	0.754	-0.267
		After first period.	0.040	0.447	0.487	
		" second "	0.081	0.166	0.247	

calcium excreted in urine and feces in the period prior to the calcium was compared to the amount excreted in the period of similar length after the injection. In one of the dogs, the calcium excretion was also determined for the next 3 days, making the period of observation 6 days after the injection.

Results.—The injection of the calcium was usually followed by irregularity of the heart action for some time, but if the injection was carried out slowly, no other ill effect was observed. But the first dog which was tried (not recorded in the experiments) died suddenly, apparently because the heart stopped beating; there were also convulsions. The other three dogs received 0.50, 0.75, and 1.10 gm. of calcium chloride, respectively. Table III and Fig. 1 show that the blood calcium increased to a considerable

¹ It is very easy to introduce a rectum or an ordinary stomach tube to about 50 to 60 cm. from the anus in a dog.

extent, dependent on the amount administered, but decreased rapidly again. After 4 to 5 hours the calcium was down to the normal again in the first two dogs, while in the last dog (Dog 5) some calcium was still retained in the blood after 6 hours. The phosphates increased moderately after the injection. There were no other characteristic changes, the acid-base equilibrium being practically undisturbed by the injection.

The results of the analyses of the calcium excretion before and after the injection are seen from Table IV. They show the peculiar result that in both dogs there was less calcium excreted in the 3 day period after the injection than before and in Dog 5 still less in the next 3 days.

DISCUSSION.

The results of the calcium ingestion experiments corroborate the findings of previous investigators (1, 2, and 3) that calcium chloride produces an increase in the concentration of chlorine and a decrease in bicarbonate of serum when given orally in large doses, and they show that a severe uncompensated acidosis with loss of base and increase of fixed acid may be produced with pH values of 7.13 in a dog and 7.14 in a human being. They also show that the blood under such circumstances is able to increase its concentration of calcium a little above the normal (Dog 2, Table I). In the two cases of Bright's disease, however, there was no increase in the blood calcium, though in one of the cases there was an extreme acidosis. It has been shown in this hospital (15) that the low blood calcium in cases of Bright's disease without phosphate retention may be attributed to the low plasma protein. The observations of the present experiments show that it is as hard to increase the blood calcium of these low protein cases as it is to increase the normal blood calcium, apparently because the protein concentration is the dominating factor, which determines the upper limit for the amount of calcium the blood is able to dissolve. In parathyroid tetany, however, the low blood calcium can be raised to the normal by the oral administration of calcium lactate (16), which shows the difference in the nature of the calcium drop in this condition from that in the low protein types of Bright's disease (17).

The absence of any clinical signs of acidosis, *e.g.* dyspnea, both in the dogs and in the human subjects, is hard to explain on the basis of the theory that the pH of the blood is the main regulator of the respiration. It will be seen from Tables I and II that the CO₂ tension remained practically constant in all the instances in which pH was reduced, while in the case of chronic nephrosis the pH was unaltered and the CO₂ tension lowered.

The observation that intravenously injected calcium chloride disappears rapidly from the blood is not in agreement with the findings of Rey in 1895 (18) who, 4 days after the injection of 0.857 gm. of calcium (as the acetate) in dogs, still found the blood calcium about 100 per cent increased. Fenyvessy and Freund (19) found a substantial increase in the blood calcium a few minutes after the injection of a large dose of calcium chloride in rabbits, although they did not obtain the high values they had calculated, and they think that injected calcium leaves the blood rapidly. Heubner and Rona (20) found that calcium chloride (0.12 to 0.27 gm. per kilo of body weight) injected intravenously into cats leaves the blood in 2 hours. Several investigators have tried to find where the injected calcium is excreted. Rüdél (21) could only account for 12 to 34 per cent of subcutaneously introduced calcium in the urine and supposed the rest to be excreted by the intestines. Rey's (18) experiments seem to leave little doubt that intravenously injected calcium is excreted for the greater part in the colon, where he could account for up to 53 per cent of the injected amount after 3 days. According to Rey, the calcium is stored in some place and then slowly excreted.

Drury² observed no increase in the calcium excretion through the bile after intravenous injection of calcium chloride.

Our own experiments failed to account for the injected calcium in feces and urine; less calcium was excreted in the period following the injection than before.

As the calcium leaves the blood and does not appear in the feces or urine within 3 to 6 days, one is compelled to assume a temporary or permanent storage of the calcium in the body.

The increase in phosphates after the injection of calcium chloride seems to be a fairly constant phenomenon, both in parathyroidectomized (16) and in normal dogs.

² Drury, D. R., personal communication; paper to be published shortly.

From the experiments on the action of oral administration of calcium lactate and intravenous administration of calcium chloride it may be assumed that calcium salts relieve tetanic symptoms, independently of any action of the acid-base equilibrium. The therapeutic action of hydrochloric acid-producing substances is probably also due to calcium action as pointed out by Gamble and Ross (2), the lowering of the bicarbonate and increase in the hydrogen ion concentration of the blood evidently producing an increased calcium ion activity. For that reason calcium chloride given orally would seem to be the most favorable salt, as it acts by both its anion and cation.

Calcium chloride has recently been enthusiastically recommended as a diuretic by Blum and associates (22) who used 15 to 20 gm. a day on patients with edema of various origins. As a severe, uncompensated acidosis usually occurs, such doses should never be employed without a careful control of the acid-base equilibrium. The question whether the diuretic effect is dependent on the anion and in some way connected with the acidotic condition is as yet unsettled.

SUMMARY.

The experiments show that the ingestion of calcium chloride may produce a severe, uncompensated acidosis in dogs and human subjects with pH values of 7.13 in a dog and 7.14 in a patient suffering from acute nephritis with edema. This acidosis is due to a replacement of HCO_3 by Cl in the blood, already observed by Gamble, Ross, and Tistall (1) and by Atchley, Loeb, and Benedict (3) as the result of absorption from the alimentary tract of the Cl of CaCl_2 without the Ca. We observed also an actual loss of base from the blood and a failure to adjust the CO_2 tension to the lowered bicarbonate. The blood calcium increased 20 per cent in one dog, while it was unchanged in another.

In two cases of Bright's disease with low blood calcium and low protein, calcium lactate, given orally, produced no characteristic change in the blood.

The intravenous administration of 0.50 to 1.10 gm. of calcium chloride had no effect on the acid-base equilibrium of the blood, but caused a moderate rise in the phosphates. The injected calcium left the blood in 3 to 6 hours. There was no increase in the daily excretion of calcium in urine and feces over a period of 6 days following the injection.

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THE NUTRITIONAL REQUIREMENTS OF BABY CHICKS.

IV. THE CHICKS' REQUIREMENT FOR VITAMIN A.*

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When Sugiura and Benedict (1) concluded from their work with pigeons and squabs that vitamin A was not required for avian nutrition, a fundamental distinction, if true, in the nutritional requirement of birds and mammals was made. It would seem, of course, that such an observation could not go unchallenged for any length of time, especially when the entire gamut of avian life was included. Consequently, it was no surprise when a little later Emmett and Peacock (2) reinvestigated this point and produced evidence unfavorable to the sweeping conclusion of Sugiura and Benedict, but in support of the view that vitamin A is required by the chicken, which is as distinctly avian as is the pigeon.

In our experiments on the nutritional requirements of baby chicks we have had occasion to accumulate considerable data bearing on this same point and in this paper we desire to present such data, all of which is in support of the early conclusion of Emmett and Peacock that the chick does require vitamin A for its existence.

In early work with baby chicks on the use of synthetic diets (3) such as are employed with success in rat nutrition we experienced varying degrees of success. A diet carrying 37 per cent of

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dextrin, 5 per cent of a salt mixture (3), 18 per cent of purified casein, 15 per cent of dried yeast, 15 per cent of butter fat, and 10 per cent of paper did prove successful in a single trial, but in other experiments where the paper was displaced by charcoal, agar, or dirt no such success in growth followed. With increasing knowledge of the liberal demands by this species of the fat-soluble vitamins such as are contained in cod liver oil (4) it was altogether probable that our many failures and rare successes in the growth of baby chicks under confinement and on synthetic diets was to be related to the low and variable supply of the fat-soluble vitamins in the butter fats used. Further, we had no knowledge of the relation of light to the growth of this species (5) at the time of these earlier experiments, although these experiments were conducted in the basement of the poultry building where the windows were often left open and with the possibility that sufficient illumination had influenced the results in some of our more successful growth experiments. All of which serves to emphasize how slow was our progress in the development of specific knowledge of the factors operating in chicken nutrition.

With more complete knowledge of the requirements of the baby chick for growth, synthetic rations were again attempted, but in which the butter fat was displaced by cod liver oil in the proportion of 5 per cent and also with saponified cod liver oil equivalent in cod liver oil to 5 per cent of the ration. Earlier observations from this laboratory had shown the stability of vitamin A to saponification (6) and later observations (7) showed the stability of the antirachitic vitamin to this same treatment. In addition, observations in this laboratory by Steenbock and Nelson (8) had disclosed the importance of the antirachitic vitamin or its equivalent in light as a necessary factor in growth. This important observation led to the use of ultra-violet light as an adjunct to the synthetic ration with the thought in mind that possibly growth could be secured. Ultra-violet light in 10 minute exposures per day, except Sunday, was used.

Chart I shows the results secured with these four rations; namely, the basal synthetic ration; the basal synthetic ration plus cod liver oil; the basal synthetic ration plus saponified cod liver oil; and the basal synthetic ration plus radiation. The chicks used were Barred Rocks taken at 1 day old and kept in pens

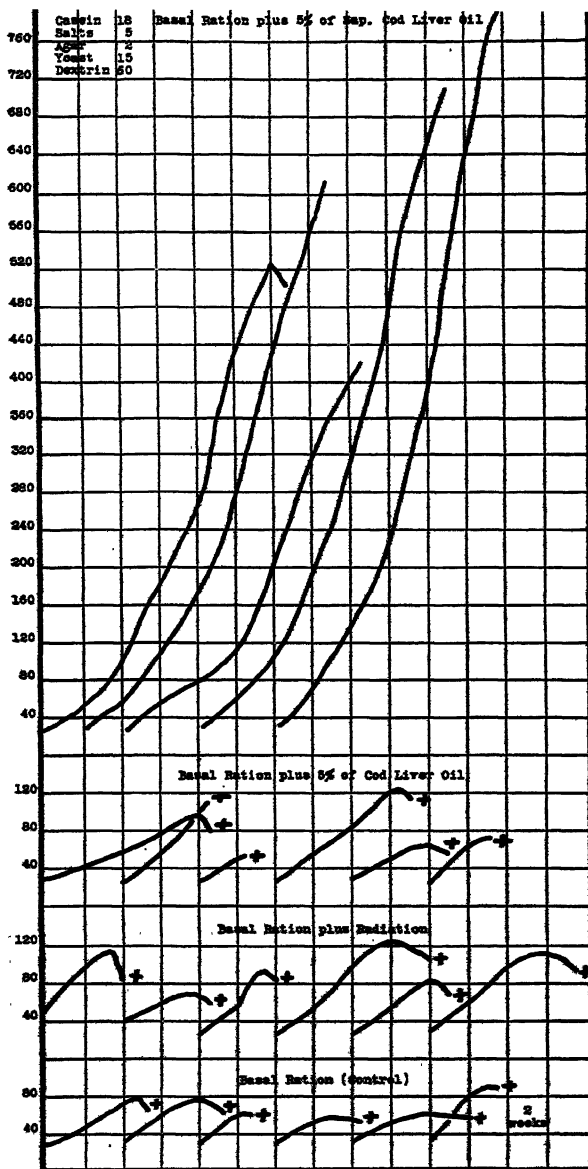


CHART I. The results secured with the basal synthetic ration; the synthetic ration plus 5 per cent of cod liver oil; the synthetic ration plus 5 per cent of saponified cod liver oil equivalent to 5 per cent of the raw oil; and the synthetic ration plus 10 minutes daily exposure (except Sunday) to the radiations from a quartz mercury lamp.

provided with shavings litter and artificial heat in the attic of our laboratory where no direct sunlight enters. They were fed the ration as dry mash and weighed weekly.

There was little or no growth on the basal synthetic ration; with the addition of 5 per cent of cod liver oil practically no better growth was secured. The cod liver oil was mixed directly into the ration which was made up weekly in order to minimize the effect of aging upon the vitamins of the oil. We have successfully prevented and cured rickets in chickens by the administration of cod liver oil, but it has usually been done by direct feeding with a pipette. Where the oil is mixed in the ration the appetite is adversely affected and consumption of the ration lags. Some individuals will consume such a ration, but in a group the results are likely to be distinctly variable. With saponified cod liver oil the results are generally much more uniform. The fat-soluble vitamins are introduced by its use, but certain objectionable smelling and tasting substances are avoided. In this group by the use of saponified cod liver oil good growth was secured as shown in Chart I, but the results are certainly not optimum for the species. The data do, however, demonstrate the favorable effect on growth in this species of the fat-soluble vitamins. Where ultra-violet light was used with the basal synthetic ration little growth was secured which indicated that this species did require vitamin A which was practically absent from the ration. With the baby chick, as with other animals, there is always a variable reserve of nutritive factors at the time of hatching which can account for some of the variations in growth secured.

The problem still confronting us was how to provide vitamin A and not provide the anti-rachitic vitamin in the ration of the chick; thereby securing more convincing evidence of the vitamin needs of this species than we had secured up to this time. In some preliminary experiments on the use of plant tissue, either green or dried, as a source of the anti-rachitic vitamin in the growth of the chick it was observed that leg weakness or rickets could not be prevented in this species by the incorporation of green plant tissue (clover) equivalent to 5 per cent of the ration or as dried plant tissue (clover) in amounts varying from 0.5 to 5 per cent of the ration. The clover used was fresh green material which had been dried in a dark attic with an electric fan. Preliminary observa-

tions on the use of green plant tissue in baby chick growth have already been published (5).

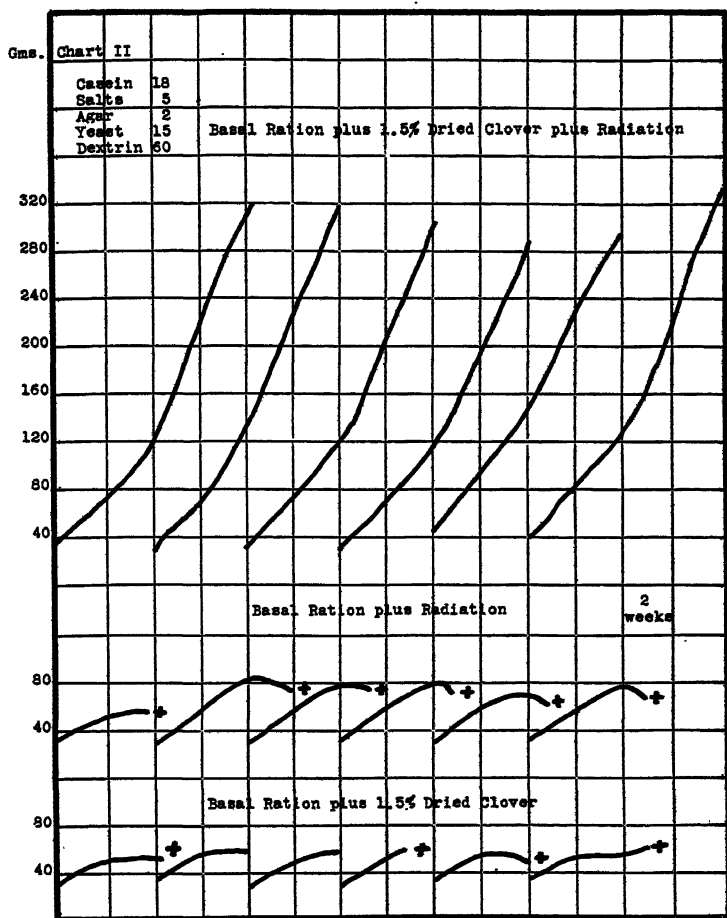


CHART II. The results secured with the basal synthetic ration plus radiation; the synthetic ration plus 1.5 per cent of dried clover; and the synthetic ration plus 1.5 per cent of dried clover plus 10 minutes daily exposure (except Sunday) to the radiations from a quartz mercury lamp. The clover displaced 1.5 per cent of dextrin.

With our further knowledge that carefully dried clover contains an abundance of vitamin A (9), but in the proportion used did not

contain sufficient of the antirachitic vitamin to protect the chick from leg weakness, it seemed possible that through the use of dried clover and ultra-violet light the problem of the vitamin A requirement of the chick could be definitely settled. Consequently, groups of 1 day old chicks (Barred Rocks) were placed upon our synthetic ration plus radiation; the synthetic ration plus 1.5 per cent of dried clover; and the synthetic ration plus 1.5 per cent of dried clover plus 10 minutes of ultra-violet light daily. The records of this experiment are shown in Chart II. According to present theory the first ration should provide all the factors of nutrition except vitamin A. Vitamin C has not been demonstrated as a requirement of the chicken; the second ration should provide all the nutritional factors except the antirachitic vitamin; and the third ration should provide all the factors and allow normal growth.

The records show that in the absence of vitamin A there was complete or early failure in the nutrition of the chick. Emmett and Peacock have reported the occurrence of ophthalmias in their chicks suffering from vitamin A deficiency, but in our many trials where vitamin A has been deficient and where death has resulted when the chick was 3 to 5 weeks of age no ophthalmias have been observed. With vitamin A deficiency in the case of the chicks we have had under observation there has been loss of appetite, extreme lethargy, and sudden death. Post-mortems have not revealed anything especially distinct although occasionally there were white streaks on the surface of the liver. Such streaks have already been observed and described by Beach (10) as occurring on the surface of the heart, liver, and spleen of chickens suffering from what he has called a "nutritional roup" and which he has attributed to a deficiency of vitamin A. Emmett and Peacock have also observed similar pathological conditions in chicks suffering from vitamin A deficiency. These white streaks have been characterized as salts of uric acid. On the other hand, Osborne and Mendel have correlated vitamin A deficiency in the diet of the rat with the occurrence of phosphatic renal calculi (11).

We do not wish to be understood as entertaining the view that vitamin A deficiency never occasions ophthalmias in the chicken. Only we have not observed it in chicks on our diets at early ages.

Possibly death intervened too early. We have seen distinct ophthalmias in older birds on rations low in their content of vitamin A, but whether it was "nutritional roup" or a roup produced by specific primary infection was not determined.



FIG. 1. No. 4654 on the left. The result of no vitamin A in the ration. The ration consisted of our basal synthetic ration composed as follows: purified casein 18, salt mixture 5, agar 2, dextrin 60, dried brewers' yeast 15, and 10 minutes exposure to ultra-violet light daily except Sunday. Weight at 5 weeks of age was 62 gm. Note the sleepy appearance of this bird.

No. 4644 on the right. The result of adding the fat-soluble vitamins (saponified cod liver oil equivalent to 5 per cent of raw oil) to the synthetic ration. Weight at 5 weeks of age was 142 gm. Good growth was secured in this group. Note the alertness of this specimen. Photographed at same age as No. 4654.

On the synthetic ration containing the 1.5 per cent of dried clover and, consequently, vitamin A, but not sufficient of the antirachitic factor for this species, the animals lingered somewhat longer than in the first group. They suffered from leg weakness and died early. With both 1.5 per cent of clover (vitamin A)

and ultra-violet light (antirachitic vitamin) imposed on the basal synthetic ration growth was very successful. Some of the birds reached weights of over 300 gm. in 8 weeks.

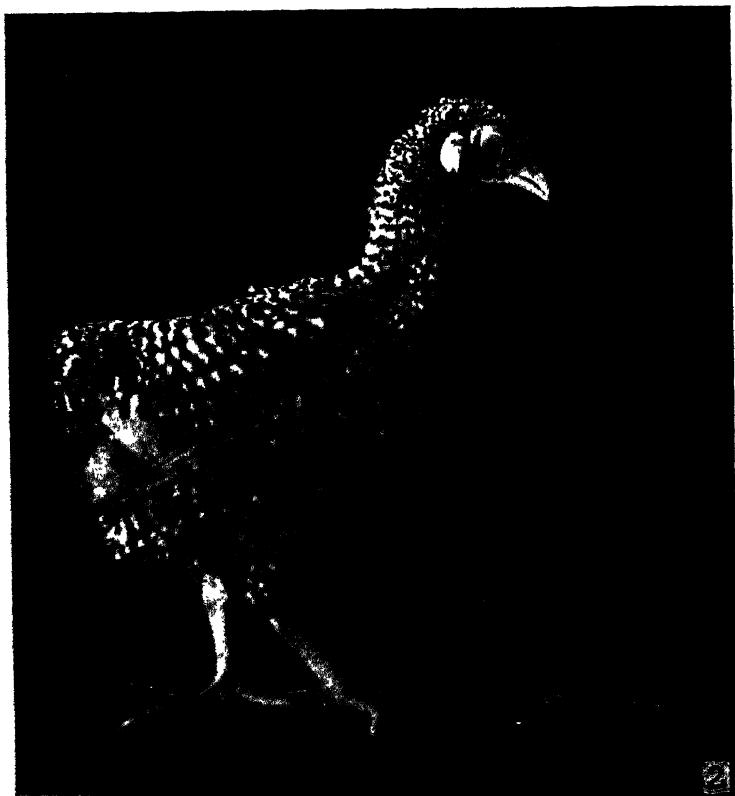


FIG. 2. The result of adding vitamin A to the synthetic ration by the use of 1.5 per cent of dried clover and in addition raying the animals 10 minutes daily, except Sunday, with the radiations from a quartz mercury lamp. Very successful growth was secured in this group. Weight at 9 weeks of age was 425 gm. This chick is one from a group of ten specimens.

In addition to the charts, pictures of three of the chicks are presented (Figs. 1 and 2). No. 4654 received the basal synthetic ration and ultra-violet light 10 minutes daily except Sunday. Vitamin A was deficient in this ration. No. 4644 received the basal ration plus saponified cod liver oil equivalent in raw oil to

5 per cent of the ration. Both vitamin A and the antirachitic vitamin were provided in this ration and good growth was secured. No. 6030 received the basal ration plus 1.5 per cent of dried clover

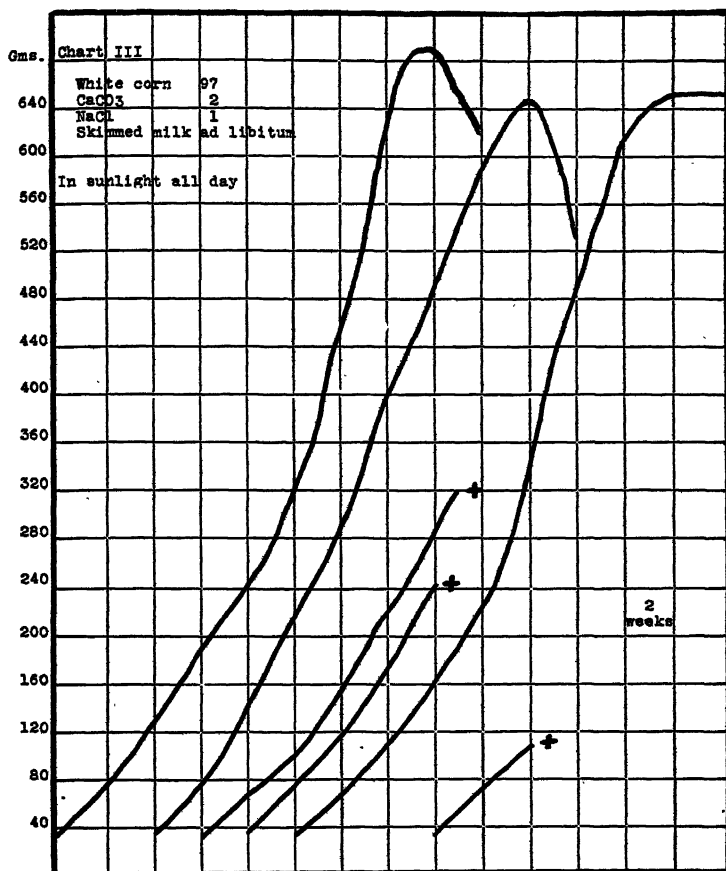
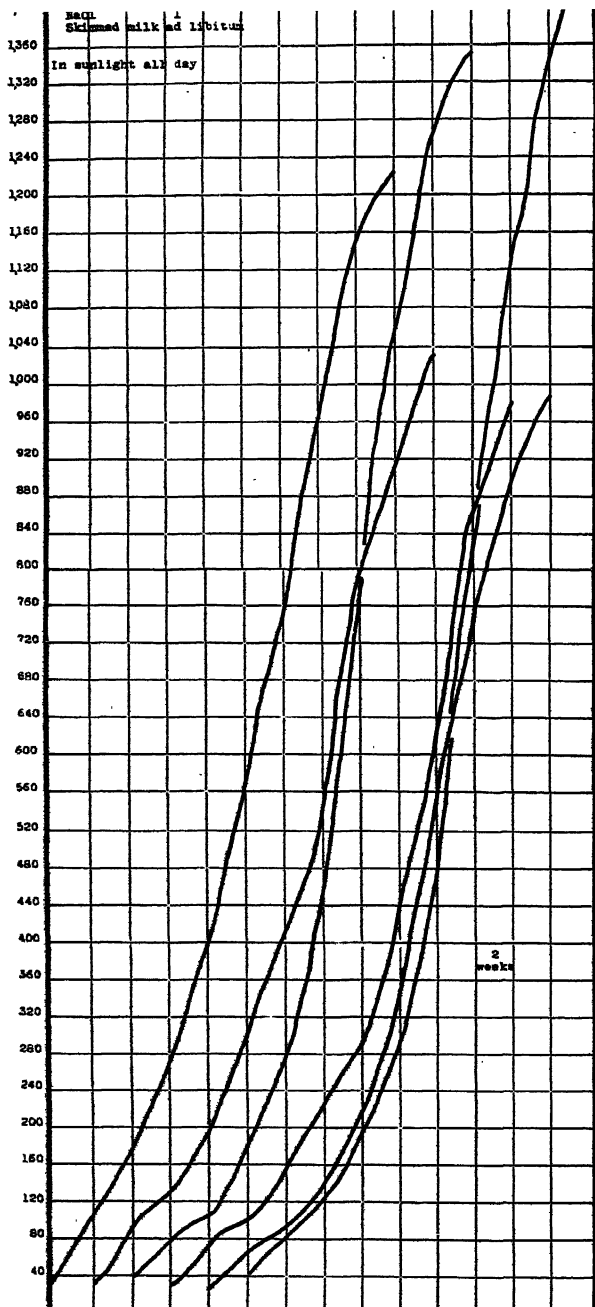


CHART III. This chart illustrates the results secured with white corn and skimmed milk. On a ration of white corn 97, calcium carbonate 2, sodium chloride 1, skimmed milk *ad libitum*, and sunlight all day, growth ultimately ceased due to the low supply of vitamin A.

plus 10 minutes radiation from a quartz mercury lamp daily, except Sunday. This ration provided vitamin A from the clover and the antirachitic factor from light and in consequence growth followed.



While the above experiments demonstrated fully the needs of vitamin A in the nutrition of the chick, additional evidence was secured through experiments involving yellow and white corn and sunlight. Some of these experiments on the influence of light on the growth of chicks and the cure of leg weakness (rickets) have already been reported (5). It was demonstrated in 1920 by Steenbock and Boutwell (12) that yellow corn was richer in its content of vitamin A than was white corn. Although at that time there



FIG. 3. The result of feeding a ration low in vitamin A. Ten birds were started in this group at 1 day old and fed a ration of 97 parts of white corn, 2 parts of calcium carbonate, 1 part of sodium chloride, and skimmed milk *ad libitum*. They were exposed to sunlight the entire day. Note the poor condition of these birds. Only three remained at the time of the photograph, the remainder having died or been used for x-rays.

had been no differentiation between vitamin A and the anti-rachitic vitamin and the relation of the latter to growth, yet the distinction between the two corns in respect to their vitamin A content still remains a fact (unpublished data from this laboratory).

In July, 1923, several groups of 1 day old chicks (White Leghorns) were placed upon white corn 97, calcium carbonate 2,

sodium chloride 1, and skimmed milk *ad libitum*. Other groups were placed upon yellow corn 97, calcium carbonate 2, sodium chloride 1, and skimmed milk *ad libitum*. The environment of the several groups differed in respect to the amount and kind of light each received. In this paper consideration is given only to those two groups receiving sunlight all day, one of which was on the *white* corn ration while the other received the *yellow* corn ration.



FIG. 4. The result of feeding a ration well supplied with vitamin A. This group of nine birds was started when 1 day old on a ration consisting of yellow corn 97, calcium carbonate 2, sodium chloride 1, and skimmed milk *ad libitum*. They were exposed to sunlight the entire day. All the birds grew normally and were splendid specimens. Three of the group had been used in x-ray work and consequently only six remained for this photograph.

On the white corn ration this nutritional factor was small although some of it was contributed by the skimmed milk. On the yellow corn ration this factor was certainly more liberally provided. The birds were in screened outdoor pens with access to heat and sunlight all day, but no extraneous sources of food. The growth records of the two groups are shown in Charts III and IV.

Those receiving the white corn ration grew considerably for a time in remarkable contrast to a group on this same ration, but not receiving sunlight. This fact has already been pointed out (5). In fact, three of the group receiving sunlight continued to grow for 14 weeks, reaching weights of over 600 gm. at which point a decline in weight set in. Nutritional failure followed and finally death. Toward the end of their lives these birds were listless and sleepy in appearance with feathers slightly ruffled

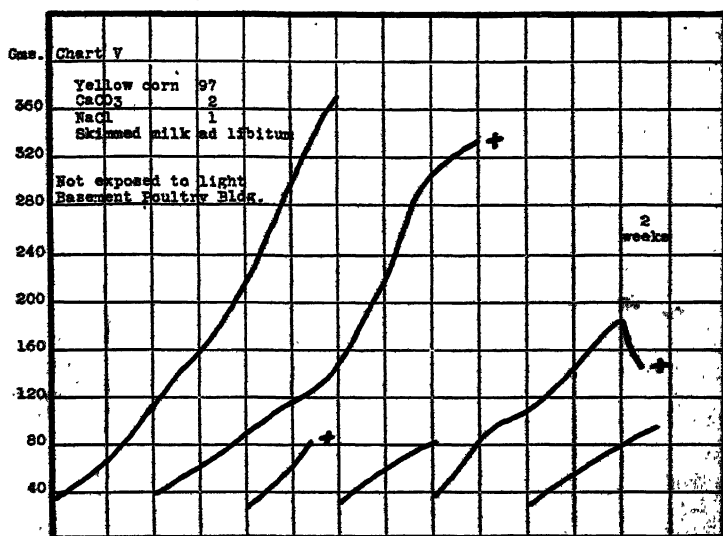


CHART V. This chart illustrates the results secured on a ration of yellow corn 97, calcium carbonate 2, sodium chloride 1, and skimmed milk *ad libitum*, but where the birds were kept out of direct sunlight and in the basement of the poultry building. The absence of the antirachitic equivalent brought on failure in continuous growth.

and soiled. There were no distinct ophthalmias in this group although an edematous condition of the eyes was seen in certain cases. The variations in growth observed in this group possibly were due to variations in the stores of reserves of vitamin A at hatching or to a difference in their ability to assimilate this factor.

Those receiving the yellow corn ration grew continuously to the termination of the experiment and the birds were judged as normal

specimens. In fact, this simple ration, supplied under summer conditions, seemed to meet the needs of this species most adequately, especially for growth. Egg production was not involved in the inquiry. We interpret the differences in growth between the two groups as due to a difference in the supply of vitamin A in the two rations. Figs. 3 and 4 illustrate in a striking manner the condition of these groups at 14 weeks of age.

Chart V is added, illustrating the failure in growth of a companion group of chicks receiving the yellow corn ration described above, but kept in the basement of the poultry building and out of the influence of direct light. In this case although there was a supply of vitamin A, the antirachitic factor was inadequately supplied and, consequently, failure of complete nutrition ultimately resulted.

SUMMARY.

In this paper experiments with growing chicks are presented showing the needs of this species for vitamin A. These results are in agreement with the observations of Emmett and Peacock and contrary to the sweeping statement of Sugiura and Benedict that "*fat-soluble vitamin is not essential in any stage of avian nutrition.*"

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A SIMPLE MICRO VESSEL WITH ELECTRODE FOR ESTIMATING THE HYDROGEN ION CONCENTRATION OF BLOOD AND OTHER BODY FLUIDS.

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In the estimation of the hydrogen ion concentration of blood and other body fluids it is frequently desirable to confirm results that have been obtained colorimetrically, and sometimes necessary, if not indispensable, to make the determination by the electrometric method. However, success with electrometric measurements is not always readily attained owing to technical and other difficulties. There is, therefore, need of further improvement of electrometric technique so as to render the method as convenient, simple, and practical as possible.

The extensive literature on the subject indicates that certain precautions are necessary with both the electrometric and colorimetric methods. In both methods the preservation of the original carbon dioxide content of the material to be examined is essential. In the electrometric method care must be used in addition, to avoid errors due to oxygen.

From a comparison of the colorimetric and electrometric methods it was pointed out by Conway-Verney and Bayliss (1) that good agreement is obtained when oxygen is kept out of the electrode vessel. In electrometric determinations of the pH of blood, errors due to oxygen may arise in two ways: first, oxygen may inadvertently be introduced into the electrode vessel; and second, the oxyhemoglobin may cause a depolarization of the hydrogen electrode. Care alone can rule out the first cause of oxygen error, but this is simpler in theory than in practice. Success in this direction would be more assured if the vessel were reduced to minimal capacity and arranged conveniently for washing readily and quickly with hydrogen. The depolarization caused by

oxyhemoglobin can be eliminated, or at least greatly reduced, by using a minimal contact between the electrode and the blood. This precaution was pointed out long ago by Michaelis (2), and has been emphasized recently by Clark (3).

It is the object of this paper to describe a micro electrode and vessel which meet the objections to shaking devices, and of interference by oxygen and loss of CO_2 , with pH results in good agreement with those obtained colorimetrically. The apparatus requires only 1 drop of fluid for the estimation, and the filling of it depends partly on capillarity of a small side tube of the vessel and partly on hydrostatic pressure of the fluid. As far as blood is concerned, minimal contact of electrode with fluid lessens the interference from oxyhemoglobin and the excellent agreement with colorimetric estimations indicates no loss of CO_2 .

Description of Electrode and Vessel.

The vessel is made from Pyrex glass tubing of 10 mm. bore and 13 mm. outside diameter. One end of the tube is drawn out into a capillary tube and bent into the shape shown in Fig. 1. This capillary tube serves for the introduction and removal of the fluid to be estimated. The length of the vessel proper is about 2 cm.; i.e., just sufficient to permit clamping to a small ring stand. Into the large end of the vessel is fitted a 2-hole rubber stopper through which are passed the connecting bridge and a small T-tube of 3 mm. bore. The T-tube accommodates a capillary tube containing the electrode and serves for the introduction of hydrogen. The electrode consists of a piece of No. 22 gauge platinum wire 1 cm. in length, sealed into a capillary tube. The capillary tube containing the electrode is passed through the T tube and is held in position by rubber tubing at the upper end. Contact with the electrode is made by means of mercury. The connecting bridge consists of a capillary tube drawn out and bent so that the tip may be pushed down into the constricted portion of the electrode vessel. The bridge is filled with saturated potassium chloride containing sufficient agar (to 3 per cent) to solidify at room temperature, the mixture being previously melted and drawn into the tube by suction and allowed to solidify.

Connection to a hydrogen generator is conveniently made by small rubber tubing with a T-tube and two small metal stop-cocks which facilitate rapid control of, and washing with, hydrogen. Stop-cock *A* connects directly with the hydrogen generator. Stop-cock *B* is momentarily opened during introduction of fluid in case it is necessary to lower the pressure in the vessel.

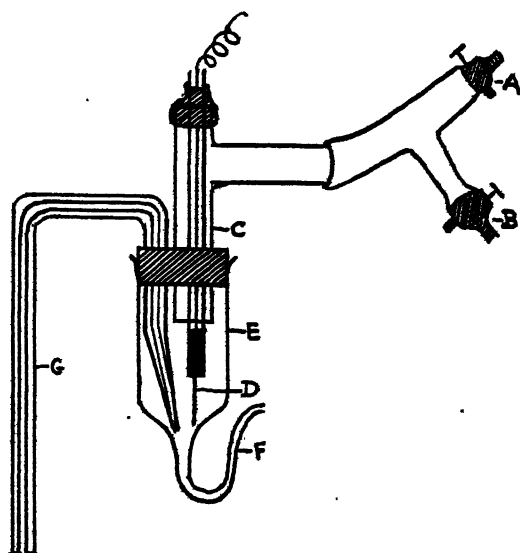


FIG. 1. Micro vessel with electrode (drawn to scale).

- A* = small metal valve to hydrogen supply.
- B* = small metal valve for release of pressure of excess hydrogen.
- C* = T-tube for support of electrode, and inlet of hydrogen.
- D* = electrode in capillary and mercury contact.
- E* = vessel.
- F* = capillary tube for introduction and removal of fluid.
- G* = connecting bridge of capillary with agar-KCl.

Use of Electrode and Vessel.

The fluid for determination of pH is collected under oil (petroleum liquidum, U. S. P.) when such a precaution is necessary (as with blood and other body fluids). After the usual preliminary flushing of the cleaned vessel with pure hydrogen, the fluid is drawn up under oil in a small bore pipette, the opening of which is of such size as to permit good contact with the opening of the

capillary *F* (see Fig. 1). It is always advisable to draw the fluid into the pipette under oil so as to obtain the necessary hydrostatic pressure which makes filling of the vessel easy. The tip of the pipette is placed in contact with the capillary of the vessel immediately after shutting off the current of hydrogen. The

TABLE I.

pH Values from Blood and Other Body Fluids by Micro Electrode and Vessel and Colorimetrically.

Fluid.	pH by micro electrode and vessel.	pH by colorimetric method.
Whole dog blood.	7.11	7.15
	7.05	6.95
	7.09	7.10
	7.19	7.20
Asphyxial dog blood.	6.82	6.80
Beef plasma.	7.17	7.15
	7.16	7.15
Horse serum.	7.28	7.30
	7.73	7.70
Human bile.	6.83	6.80
Dog bile.	6.17	6.20
	6.35	6.40
Cat.	6.09	6.10
	6.75	6.80
Ascitic fluid.	7.21	7.20
Human saliva.	6.94	7.00
Human cerebrospinal fluid.	7.19	7.20
	7.22	7.20

fluid is now allowed to flow into the vessel until it just touches the electrode. Simultaneously, contact of the fluid is made with the agar bridge which projects into the constricted portion of the vessel. All connections being properly made, measurements with a potentiometer are made immediately and as quickly as possible. Shaking of the vessel is not required. Equilibrium is

rapidly attained, permitting several check determinations to be made within 10 minutes.

The vessel is emptied by flushing with hydrogen, and cleaned, if necessary, by introducing distilled water into the capillary. When not in use, the vessel is kept filled with distilled water.

Table I illustrates a number of results obtained with whole blood, plasma, serum, cerebrospinal fluid, ascitic fluid, saliva, and bile. It is seen that agreements with colorimetric values by the method of Levy, Rowntree, and Marriott (4) are good. The colorimeter method was used with the additional precaution of a layer of liquid petrolatum over the blood within the celloidin sac and also over the dialysate.

Originally, a somewhat larger vessel had been used by us for obtaining the pH values of over 100 samples of blood and plasma from a series of 35 experiments on dogs. These values were always confirmed by the colorimetric method just mentioned. The agreements were as nearly perfect as could be expected under the conditions of the experiments which showed changes from the intravenous injections of a variety of agents, asphyxia, etc., and of which a preliminary report has been made (5).

Using the micro electrode and vessel, the following pH values were obtained with whole blood during the course of an experiment on a dog in which different degrees of asphyxia were induced by increasing the dead space with a long rubber tube attached to the trachea: Before asphyxia, 7.22 and 7.20; during mild asphyxia, 6.98; and at the end of fatal asphyxia, 6.82. After measuring these bloods, 0.05 M acid potassium phthalate gave 3.97 (theoretical). The results of this experiment show that this electrode is capable of detecting the variations in pH accompanying such physiological changes as are induced by different degrees of asphyxia, and that the results were correct because at the end of the experiment, the electrode gave the correct value with standard phthalate.

CONCLUSIONS.

1. A simple and convenient micro vessel with electrode for accurate estimation of pH of blood and other body fluids is described.

2. The micro vessel requires less than 0.1 cc., usually 0.05 cc., or roughly 1 drop, of fluid, permitting the repeated estimation of pH of blood and other body fluids of the smallest animals with impunity, and the obtaining of blood from human subjects without stasis from a finger under oil containing a few crystals of oxalate.

3. Estimations with the micro electrode and vessel are made rapidly and agree perfectly with those obtained colorimetrically, loss of CO_2 and interference by oxygen being avoided.

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4. Levy, R. L., Rowntree, L. G., Marriott, W. McK., *Arch. Int. Med.*, 1915, xvi, 389.
5. Hanzlik, P. J., De Eds, F., and Tainter, M. L., *J. Pharmacol. and Exp. Therap.*, 1923, xxi, 217.

THE URIC ACID PROBLEM.

AN EXPERIMENTAL STUDY ON ANIMALS AND MAN, INCLUDING GOUTY SUBJECTS.*

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I.

INTRODUCTION.

To this day, gout is still recognized mainly on the basis of visible urate deposits. The literature abounds in conflicting

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guesses as to the exact nature of the underlying disorder, and the total impression produced by this literature is one of confusion rather than of progress. Various combinations of circumstances doubtless are responsible for the long period of sterility in so unique and interesting a field of research, but the two main causes have been, first, the apparent inapplicability of animal experimentation, and, second, the lack of suitable analytical technique. Until all the essential facts concerning the behavior of uric acid within the normal organism have been ascertained, the uric acid metabolism in gout is likely to remain a matter of mere speculation.

The investigations recorded in this paper were begun on the basis of the conviction that we could determine uric acid in blood with a precision certainly quite unattainable in earlier work. We had no preformed ideas or working theories—other than the belief that the first thing to find out should be the behavior of administered uric acid.

The unexpected results obtained have compelled us to try to formulate interpretations. It is impossible to do continuous research without trying to correlate the facts. While we may have been rather too free with new explanations, we have endeavored to confine ourselves to theories which can be tested by further experimental work; and if we have omitted to protect ourselves by reservations, we have done so because reservations seldom serve any useful purpose and take up space—and not because we are blind to the fact that original theories are nearly always wrong.

II.

HISTORICAL REVIEW

Both Liebig and Wöhler knew that uric acid by oxidation can yield allantoin,¹ but in their joint research² (1838) they proved and emphasized the fact that uric acid can be decomposed into urea and oxalic acid, and it was this more complete decomposition, rather than the allantoin formation which became dominant in the metabolism literature of their time. In 1848 Wöhler and Frerichs³ "proved" by intravenous injections of uric acid into dogs, and by mouth feeding with man, that the animal

¹ Ostwald, W., *Grosse Männer*, Leipsic, 2nd edition, 1919, i.

² Wöhler, F., and Liebig, J., *Ann. Chem.*, 1838, xxvi, 241.

³ Wöhler, F., and Frerichs, F. T., *Ann. Chem.*, 1848, lxx, 335.

organism converts the nitrogen of uric acid into urea. Their results were later "confirmed" by Neubauer⁴ as well as by Zabelin,⁵ and for a long time it was considered as settled that uric acid when introduced into the animal organism is excreted as urea. The occurrence and possible significance of allantoin necessarily remained unknown, since its determination or qualitative isolation from urine remained an unsolved problem.

It may be noted here that the ammoniacal silver precipitation of uric acid was first introduced by Salkowski⁶ in 1871 and that it was only after the development of this process into the classical Salkowski-Ludwig method⁷ that dependable uric acid determinations in urine became possible. Up to that time Heinz's method for uric acid, and Liebig's titration method for urea were used. It is also to be noted that up to the end of the last century protein metabolism, like the metabolism of fats and carbohydrates, was looked upon as involving only oxidations, and the excretion of uric acid signified incomplete or delayed oxidation. Thus a great number of investigations were published intending to show an increased uric acid output in man under conditions implying diminished oxidation, such as impaired respiration, severe cyanosis, carbon monoxide poisoning, and others (Bartels⁸). The fundamental difference between the end-products of the protein metabolism as found in birds and reptiles on the one hand, and mammals on the other, was interpreted as reflecting incomplete oxidations in animals which "seldom drink."⁹

The first doubt as to the correctness of this deficient oxidation hypothesis was raised by the results obtained by Cech¹⁰ in Salkowski's laboratory (1877). These results showed that urea when fed to hens is not recovered as urea. Hans Meyer and Jaffé¹¹ in the same year showed that the administered urea is excreted mainly in the form of uric acid. The theory that uric acid is the result of deficient oxidations within the body was definitely replaced by the modern view of the synthetic origin of uric acid in birds when Minkowski,¹² in 1886, showed that the uric acid dis-

⁴ Neubauer, C., *Ann. Chem.*, 1856, xcix, 206.

⁵ Zabelin, *Ann. Chem.*, 1862-63, suppl. 2, 326.

⁶ Salkowski, E., *Virchows Arch. path. Anal.*, 1871, lii, 58; *Arch. Physiol.*, 1872, v, 210.

⁷ Ludwig, E. L., *Anz. Akad. Wissensch. Math.-naturw. Cl., Wien*, 1881, xviii, 92. Salkowski, E., and Leube, W. O. L., *Die Lehre vom Harn*, Berlin, 1882. Ludwig, E. L., *Wien. med. Jahrb.*, 1884, 597. Salkowski, E., *Z. physiol. Chem.*, 1890, xiv, 31.

⁸ Bartels, K. H. C. B., *Deutsch. Arch. klin. Med.*, 1866, i, 13.

⁹ von Liebig, Justus, *Animal chemistry or organic chemistry in its application to physiology and pathology*, Cambridge, 1842.

¹⁰ Cech, C. O., *Ber. chem. Ges.*, 1877, x, 1461.

¹¹ Meyer, H., and Jaffé, M., *Ber. chem. Ges.*, 1877, x, 1930. Meyer, H., *Beiträge zur Kenntniss des Stoffwechsels im Organismus der Hühner*, Dissertation, Königsberg, 1877.

¹² Minkowski, O., *Arch. exp. Path. u. Pharmacol.*, 1886, xxi, 41; 1893, xxxi,

appeared almost completely from the urine of geese after extirpation of the liver.

Fundamental work by Miescher and Kossel in the 70's and early 80's had in the meantime furnished a chemical foundation for a more definite interpretation of the metabolic origin of uric acid. Horbaczewski¹³ first demonstrated the formation of uric acid from nucleic materials by *in vitro* experiments (with spleen pulp), but he unfortunately also side-tracked subsequent investigations by his hypothesis of dead leucocytes being the only precursors of the uric acid. The origin of this hypothesis from earlier observations¹⁴ of high uric acid excretion in leukemia is obvious enough, but the unconditional linking together of leucocytosis and uric acid excretion was a mistake.¹⁵

Numerous investigations showing greatly increased uric acid excretion by man after feeding thymus, pancreas, or liver were carried out in 1895 and 1896 (Weintraud, Umber, and others¹⁶), and these proved that Horbaczewski's view of leucocytosis after meals as the cause of the extra uric acid excretion could not be correct. A clear understanding of the different sources of the uric acid in man was presented by Burian and Schur,¹⁷ who introduced and successfully developed the concepts of endogenous and exogenous uric acid (1900-06). Burian and Schur concluded from their own experiments, as well as from data gathered from the literature, that the endogenous uric acid output in any given individual has a constant value, that the value is different for different individuals, and, finally, that within wide limits the value is independent of the protein content of the food. Sivén,¹⁸ independently, obtained similar results about the same time, and for much wider variations in the protein content of the food (21 to 2.8 gm.).

As often happens in connection with pioneer research, so here the essential conclusion was overdrawn. That the endogenous uric acid excretion is influenced to a considerable, though variable, extent by the protein content of the food was shown (1905) by Folin.¹⁹ Folin's findings by no means destroyed the validity of the endogenous uric acid concept of Burian and Schur, but they showed that some unknown modifying factors are

¹³ Horbaczewski, J., *Monatsh. Chem.*, 1889, x, 624; 1891, xii, 221.

¹⁴ Ranke, H., *Beobachtungen und Versuche über die Ausscheidung der Harnsäure beim Menschen im physiologischen Zustande und in einigen Krankheiten*, München, 1858. Jacobasch, H., *Virchows Arch. path. Anat.*, 1868, xliii, 196. Salkowski, E., *Virchows Arch. path. Anat.*, 1870, l, 174.

¹⁵ Richter, P. F., *Z. klin. Med.*, 1895, xxvii, 290.

¹⁶ Weintraud, W., *Berl. klin. Woch.*, 1895, xxxii, 405. Umber, F., *Z. klin. Med.*, 1896, xxix, 174.

¹⁷ Burian, R., and Schur, H., *Arch. Physiol.*, 1900, lxxx, 241; 1901, lxxxvii, 239; 1903, xciv, 273. Burian, R., *Med. Klin.*, 1905, i, 131; 1906, ii, 479, 514, 540.

¹⁸ Sivén, V. O., *Skand. Arch. Physiol.*, 1901, xi, 123.

¹⁹ Folin, O., *Am. J. Physiol.*, 1905, xiii, 66.

involved. Mareš²⁰ and Smetánka²¹ attempted to designate these factors on the basis of increased activity on the part of the digestive glandular organs in response to protein food. More recently, Lewis²² and associates have advanced the view that the increased uric acid which they have obtained after feeding amino acids may be a consequence of the high specific dynamic action of these products. Through all the more recent literature on endogenous uric acid the variations in the excretions have been interpreted on the basis of corresponding variations in the production.

The factors governing the production and excretion of exogenous uric acid have been subjected to much investigation. Here, as in the case of the endogenous uric acid, the actual results obtained from metabolism experiments have rarely been in satisfactory agreement with the underlying theoretical considerations. Weintraud¹⁶ (1895) noticed the disproportion between the amounts of purines fed in the form of calves' thymus and the amounts of uric acid excreted. Minkowski,²³ after feeding 3 gm. of hypoxanthin to a normal person recovered 49 per cent in the form of uric acid. Burian and Schur similarly obtained only 46 per cent recovery of urinary purines after taking 1.6 gm. of hypoxanthin. Burian and Schur made many laborious analyses of the purine contents of different food materials in order to learn how much was excreted. The validity of these tissue analyses²⁴ was questioned by Loewi²⁵ and again verified by Burian and Hall.²⁶

Burian and Schur made many feeding experiments with liver, spleen, beef, veal, and bacon, and recorded an average purine recovery of about 50 per cent in terms of the "purine content" of the food. From their own data, as well as those of others,^{27,28} representing thirteen experiments with six different subjects, they concluded that, of absorbed purine material, a large fraction disappears and about 50 per cent is normally recovered as uric acid. Burian and Schur undoubtedly laid too much stress on this figure, for some of their own results were quite different. With two different individuals they recovered only about 25 per cent.

²⁰ Mareš, F., *Arch. Physiol.*, 1910, cxxxiv, 59.

²¹ Smetánka, F., *Arch. Physiol.*, 1911, cxxxviii, 217.

²² Lewis, H. B., and Doisy, E. A., *J. Biol. Chem.*, 1918, xxxvi, 1. Lewis, H. B., Dunn, M. S., and Doisy, E. A., *J. Biol. Chem.*, 1918, xxxvi, 9. Lewis, H. B., and Corley, R. C., *J. Biol. Chem.*, 1923, lv, 373.

²³ Minkowski, O., *Arch. exp. Path. u. Pharmacol.*, 1898, xli, 375.

²⁴ Burian, R., and Schur, H., *Z. physiol. Chem.*, 1897, xxiii, 55.

²⁵ Loewi, O., *Arch. Physiol.*, 1902, lxxxviii, 296.

²⁶ Burian, R., and Hall, J. W., *Z. physiol. Chem.*, 1903, xxxviii, 336. Hall, I. W., *The purin bodies of food stuffs and the rôle of uric acid in health and disease*, London, 2nd edition, 1903.

²⁷ Krüger, M., and Schmid, J., *Z. physiol. Chem.*, 1901-02, xxxiv, 549.

²⁸ Kaufmann, M., and Mohr, L., *Deutsch. Arch. klin. Med.*, 1902, lxxiv, 157, 348, 586.

Schittenhelm, a persistent advocate of the destruction theory, in experiments with Frank,²⁹ fed sodium nucleate (from thymus nucleic acid) to three subjects. The recovery, in the form of urinary uric acid, was 5, 10, and 41 per cent, respectively. Dohrn,³⁰ in a similar experiment with thymus nucleic acid recovered 11 per cent from a normal individual. Rother,³¹ in experiments with yeast nucleic acid in two practically normal subjects recovered 12 and 36 per cent; these figures, however, are minimum figures, since Rother did not follow up the experiments long enough to get the uric acid output back to the original levels.

The inevitable weak point in all feeding experiments is the uncertainty about the extent of absorption, and the form in which absorption takes place. This is well illustrated in these experiments. Frank and Schittenhelm's cases, with the low recovery, showed before the beginning of the experiment a feces nitrogen of more than 20 per cent of the urinary nitrogen, indicating some intestinal disturbance. In these cases most of the phosphorus of the nucleic acid was recovered from the feces, contrary to what happened in their third case with the higher recovery, where most of the phosphorus given was found in the urine. The same considerable increase in the phosphorus of the feces was found by Dohrn, who concluded (as Schittenhelm might have done) that intestinal destruction of most of the nucleic acid given, was the cause of the low urinary recovery.

Sivén,³² like Burian and Schur, registered a recovery of about 50 per cent of the food purines, the purines having been taken as 1,000 cc. of beef broth a day, containing a purine quantity equal to Sivén's own endogenous uric acid output. Sivén went on to prove that the loss occurred in the intestinal canal, in other words that there was no loss in the strict metabolic sense of the word. One of the few unanimous results obtained by all investigators is that the feces never contain more than traces of purine bodies. Likewise there is an agreement that the pancreatic or intestinal juice does not break up the purine ring. Sivén³³ reentered a road opened by Baginsky³⁴ in 1884 and now showed that *Bacterium coli* in test-tube experiments in 24 to 48 hours split considerable quantities of purines (beef broth). The degree to which this breakdown takes place was studied by Thannhauser and Dorfmueller,³⁵ who showed that the bacterial flora of the human intestines metabolizes the nitrogen of nucleosides into ammonia. In 20 days' experiments, 70 to 100 per cent were

²⁹ Frank, F., and Schittenhelm, A., *Z. physiol. Chem.*, 1909, lxxii, 269. Brugsch, T., and Schittenhelm, A., *Der Nukleinstoffwechsel und seine Störungen*, Jena, 1910.

³⁰ Dohrn, M., *Z. physiol. Chem.*, 1913, lxxxvi, 130.

³¹ Rother, J., *Z. physiol. Chem.*, 1921, cxiv, 149.

³² Sivén, V. O., *Arch. Physiol.*, 1912, cxlv, 283.

³³ Sivén, V. O., *Arch. Physiol.*, 1914, clvii, 582.

³⁴ Baginsky, A., *Z. physiol. Chem.*, 1883-84, viii, 395.

³⁵ Thannhauser, S. J., and Dorfmueller, G., *Z. physiol. Chem.*, 1918, cii, 148.

metabolized. Rother,³¹ in experiments with yeast nucleic acid and human feces in test-tubes, confirmed the ammonia formation and showed that the destruction after 40 to 48 hours in a thermostat represented about one-half of the nucleic acid originally present.

The main conclusion to be drawn from the results reported in these different papers is that the intermediary destruction of uric acid in man cannot be definitely proved (or disproved) by means of feeding experiments.³⁶

It is interesting to note that Burian and Schur actually fortified their theory of uric acid destruction in man by two hypodermic uric acid injections, especially since other investigators have "proved" the indestructibility of uric acid in the human organism by means of similar experiments.

Before citing the literature which has been accepted as proving that man does not destroy uric acid, it is necessary to refer to the history of allantoin, for the newer developments are based to a large extent on allantoin findings.

In 1876, Salkowski^{37,38} fed solid uric acid to dogs, and showed that a part of the uric acid was excreted in the form of allantoin. Salkowski also found allantoin in the urine of two meat-fed dogs, but as he was unable to find it in the urines of seven other meat-fed dogs he failed to recognize that allantoin is a normal constituent of dog's urine. Salkowski's discovery remained isolated if not forgotten, until Minkowski,³⁹ in 1898, again discovered allantoin in the urine of dogs—after feeding the animals with calves' thymus. Minkowski was unable to find the allantoin when horse meat was substituted for thymus. On feeding allantoin to man and to dogs, he recovered in the urine about 70 per cent in the case of the dogs, as against only about 20 per cent in man, and on the basis of these findings Minkowski tentatively concluded that the decomposition of allantoin, with urea formation, occurs more readily in man than in dogs. Minkowski's findings were confirmed by Cohn,³⁹ Salkowski,⁴⁰ Poduschka,⁴¹ Mendel,⁴² and others. These findings necessarily raised the question whether and to what extent allantoin occurs in urine as one of the end-products of purine metabolism (Burian¹⁷). But it was only after Wiechowski had devised a method for at least approximately dependable determinations

³⁶ Results, supposed to indicate an intestinal destruction of endogenous uric acid, have recently been issued by H. Steudel (Steudel, H., *Z. physiol. Chem.*, 1922-23, cxiv, 267). There is no doubt that Steudel, working with not quite fresh urines, turned over to him by Rubner, has encountered and failed to interpret the not uncommon disappearance of uric acid in urines on standing.

³⁷ Salkowski, E., *Ber. chem. Ges.*, 1876, ix, 719.

³⁸ Salkowski, E., *Ber. chem. Ges.*, 1878, xi, 500.

³⁹ Cohn, T., *Z. physiol. Chem.*, 1898, xxv, 507.

⁴⁰ Salkowski, E., *Centr. med. Wissensch.*, 1898, xxxvi, 929.

⁴¹ Poduschka, R., *Arch. exp. Path. u. Pharmacol.*, 1900, xlv, 59.

⁴² Mendel, L. B., and Brown, E. W., *Am. J. Physiol.*, 1899-1900, iii, 261.

of small as well as of large amounts of allantoin in urine (1907) that the researches began to yield consistent results. Wiechowski⁴³ was able to show that allantoin is a constant product of the endogenous metabolism of the dog, cat, rabbit, and monkey, and that the allantoin excretion on purine-free diets exhibits about the same degree of uniformity as Burian and Schur had found for the endogenous excretion of uric acid in man.

In the course of his endeavors to prove that allantoin in the urines of animals has the same origin and significance as the uric acid in man, Wiechowski^{44,45} took particular pains to isolate and identify the small amount of allantoin excreted by man on purine-free diets. The daily allantoin excretion in man amounted to only 12 to 14 mg., and was no greater in leukemia (one case) or in gout (two cases).

The most extensive investigations based on the concept of the identical origin and significance of uric acid and allantoin are those of Hunter⁴⁶ and his coworkers. They have determined the total purines, the uric acid, and the allantoin in the urines of a large series of different animals, and have introduced the term uricolytic index to express the per cent of uric acid nitrogen plus allantoin nitrogen represented by allantoin. This index varies between 80 and 98 for the different animals investigated.

The fact that endogenous allantoin, at least in part, has the same metabolic origin and significance as uric acid is important enough; but from this fact it does not necessarily follow that the uric acid which is destroyed within the animal organism is quantitatively excreted in the form of allantoin. It would appear that Wiechowski adopted the view of such quantitative transformation and excretion mainly because he found that in uricolysis experiments with liver extracts the uric acid is quantitatively converted into allantoin. This observation, coupled with the fact that uricolysis can be obtained with tissues of animals which normally excrete allantoin, but not with human tissues is, of course, suggestive (Wiechowski,⁴⁷ Jones,⁴⁸ and Schittenhelm⁴⁹).

But there is room for skepticism as to the true significance of such experiments, and the interpretations drawn from them must be confirmed by means of real metabolism experiments. The recorded metabolism

⁴³ Wiechowski, W., *Beitr. chem. Physiol. u. Path.*, 1908, xi, 109.

⁴⁴ Wiechowski, W., *Biochem. Z.*, 1909, xix, 368.

⁴⁵ Wiechowski, W., *Biochem. Z.*, 1910, xxv, 431.

⁴⁶ Hunter, A., and Givens, M. H., *J. Biol. Chem.*, 1912-13, xiii, 371; 1914, xvii, 37. Hunter, A., *J. Biol. Chem.*, 1914, xviii, 107. Hunter, A., Givens, M. H., and Guion, C. M., *J. Biol. Chem.*, 1914, xviii, 387. Hunter, A., and Givens, M. H., *J. Biol. Chem.*, 1914, xviii, 403. Hunter, A., and Ward, F. W., *Trans. Roy. Soc. Canada*, 1919, xiii, sect. 4, 7.

⁴⁷ Wiechowski, W., *Arch. exp. Path. u. Pharmacol.*, 1909, lx, 185.

⁴⁸ Winternitz, M. C., and Jones, W., *Z. physiol. Chem.*, 1909, lx, 180.

Miller, J. R., and Jones, W., *Z. physiol. Chem.*, 1909, lxi, 395.

⁴⁹ Schittenhelm, A., *Z. physiol. Chem.*, 1909, lxi, 248.

experiments bearing on the subject may be divided into three different kinds, namely:

(a) Subcutaneous or intramuscular injections of urate solutions into animals—which should yield approximately 100 per cent recovery in the form of much allantoin plus a little uric acid. (Under (a) may be included the few experiments with allantoin which are less important, but should prove the indestructibility of allantoin in the animal as well as in the human organism.)

(b) Subcutaneous injections of uric acid into human subjects. These should yield approximately 100 per cent recovery in the form of extra uric acid. (Under (b) may be included similar experiments with definite precursors of uric acid.)

(c) Intravenous injections of uric acid in man, which should also yield approximately complete recovery in the form of uric acid alone.

(a) Wiechowski⁴⁸ seems to have made only a couple of subcutaneous uric acid injections in animals, probably because uric acid so administered is distinctly toxic and this produces confusing results.

To a fasting dog he gave 600 mg. of uric acid in the form of sodium urate. The recovery in 24 hours was 104 per cent, of which 24 per cent was uric acid and the rest allantoin. This apparently excellent result is somewhat impaired by the marked toxic effects accompanied by a material increase in the total nitrogen excretion.

From a similar subcutaneous injection of urate into a rabbit, Wiechowski recovered only 56 per cent.

Hunter and Givens⁴⁹ have made similar experiments (with a monkey). In their first experiment they injected subcutaneously 40 mg. per kilo of body weight. The effects of the urate, both general and local, were very severe. Extensive necrosis occurred at the place of the injection. The uric acid plus allantoin recovery, if extended over a period of 8 days, would have been 100 per cent, but the authors properly concluded that the experiment was valueless for such purposes. In two other similar experiments, with 20 and 30 mg. of uric acid per kilo, they recovered 40 to 50 per cent of the uric acid as such and obtained very little extra allantoin.

These experiments taken all together constitute a rather meager confirmation of the view that administered uric acid must be excreted as a mixture of uric acid and allantoin.

The excretion of allantoin in response to subcutaneous injections of allantoin in man was studied by Wiechowski (three experiments). His best recovery was 74 per cent. Hunter and Givens from three similar injections of allantoin in a monkey recovered from 75 to 90 per cent. Injections of allantoin produced none of the toxic symptoms obtained from uric acid.

(b) We have found the records of six hypodermic injections of uric acid into human subjects. Two of these were made by Burian and Schur

⁴⁸ Hunter, A., and Givens, M. H., *J. Biol. Chem.*, 1914, xvii, 37.

and have already been mentioned. They gave a recovery of about 50 per cent. Of the other four, two were made by Soetbeer and Ibrahim,⁵¹ and two by Wiechowski.⁴⁷

Soetbeer and Ibrahim dissolved the uric acid by means of piperazine. In the first subject they injected 860 mg. of uric acid. During the day of the injection the increase of the uric acid elimination above the previous level corresponded to 75 per cent of the uric acid administered. The experiment had to be discontinued after this 1st day, the subject refusing further cooperation. Ibrahim then received 1,260 mg. and excreted above the average previous output 81 per cent during the 1st day, 18 per cent during the 2nd day, another 28 per cent extra during the 3rd day; and during the 4th and 5th days, taken together, another 45 per cent, making all together an extra elimination of about 170 per cent. At that time the output was not yet back to normal, but the experiment was discontinued since the condition of the subject "did not make a further continuation advisable." At that same time the nitrogen output had risen from a previous average of 23.1 to 26.3 gm. a day. It is quite evident that no conclusion concerning the destruction or non-destruction of uric acid in man can be drawn from these experiments. There is no doubt about an extra, what we may call "toxic," uric acid elimination playing a confusing rôle in the experiment.

Wiechowski injected hypodermically into himself in two experiments 990 and 440 mg. of uric acid, respectively, both times in the form of sodium urate. The recovery after the first injection amounted to 82 per cent, distributed over 3 days. From the second experiment Wiechowski recovered 50 per cent during the 1st day, the 2nd day showed a lower output than any day during the control period, and the 3rd day a slightly elevated figure. We do not think it correct to add this to the recovery figure from the 1st day, as Wiechowski did. His total recovery was 61 per cent.

An intramuscular injection into a gouty man of 500 mg. of uric acid dissolved in piperazine (von Benczur,⁵² in Brugsch's Clinic) followed by fever up to 39°C. and very marked general reaction for 2 days demonstrates clearly the toxic effect of the injection, and the figures allow no certain conclusion about the degree of recovery of the uric acid.

Subcutaneous or intramuscular injections of adenosine and guanosine have proved just as unsatisfactory as the uric acid injections for demonstrating the indestructibility of uric acid. Such injections were first used by Thannhauser and Bommès.⁵³ Rother,⁵⁴ at that time working with Brugsch, presented an adequate criticism of some of the recovery calculations of Thannhauser and Bommès, and, after the presentation of new experimental material, concluded that the nucleoside injections of-

⁵¹ Soetbeer, F., and Ibrahim, J., *Z. physiol. Chem.*, 1902, xxxv, 1.

⁵² von Benczur, G., *Z. exp. Path. u. Therap.*, 1909-10, vii, 339.

⁵³ Thannhauser, S. J., and Bommès, A., *Z. physiol. Chem.*, 1914, xci, 336.

⁵⁴ Rother, J., *Z. physiol. Chem.*, 1920, cx, 245.

ferred no support for the indestructibility theory. Later and more extensive experiments by Thannhauser and Schaber⁵⁵ do not seem to disprove the conclusion of Rother, as can be seen from the subjoined table which contains the results presented in the three papers just mentioned.

Table Showing Uric Acid Excretion from Subcutaneous or Intramuscular Injections of Adenosine and Guanosine.

Purine compound.	Subject.	Recovery (uric acid). <i>per cent</i>	Author.	Remarks.
Adenosine.	Normal.	64	Thannhauser.	Thannhauser claimed 82 per cent recovery. See Rother's criticisms.
"	"	Nothing.	Rother.	Vomiting; heart symptoms.
"	"	120	Thannhauser.	Fever. Uric acid above normal at the end of the experiment.
"	"	89	"	
"	"	117	"	No return to normal excretion.
"	"	50	"	Thannhauser claimed recovery of 61 per cent.
"	"	40	"	Thannhauser claimed recovery of 47 per cent.
"	Gout.	70	"	Thannhauser claimed recovery of 85 per cent.
"	"	Nothing.	"	
Guanosine.	Normal.	76	"	
"	"	126	"	Slight fever. Excretion greater the 2nd day than on the 1st.
"	"	18	Rother.	
"	?	54	"	
"	"	79	"	Fever.
"	Arthritis.	53	"	"
"	Normal.	101	Thannhauser.	
"	"	36	"	
"	Gout.	120	"	Gout attack.
"	"	0	"	

⁵⁵ Thannhauser, S. J., and Schaber, H., *Z. physiol. Chem.*, 1921, cxv, 170.

Injections of nucleosides have also been undertaken by Gudzent, Wille, and Keeser⁵⁶ to support the indestructibility theory, and by Schittenhelm and Harpuder⁵⁷ to tear it down. The striking feature is the wide variability of the recovery results, ranging between zero and more than 120 per cent for the adenosine, and between 18 and 126 per cent for the guanosine. Another factor to which attention is called is the toxicity of the products demonstrated not only by high temperatures but, for adenosine, also by vomiting and symptoms from the heart (Rother). Another toxic symptom is the long continued elevation of the uric acid output in two of the adenosine injections, where the experiments were discontinued before the normal level was reached, with recovery of about 120 per cent.

(c) Finally, we have the *intravenous* injection of uric acid, introduced by Umber, in 1910. Umber and Retzlaff⁵⁸ used 500 mg. of uric acid dissolved by means of 1 gm. of piperazine in 30 cc. of water. From results obtained by this method Umber concluded that the normal individual does not destroy uric acid. This statement of Umber has been widely accepted. It is therefore important to state that it seems to have been based upon the results from two individuals, one of whom excreted 80 per cent in 4 days, the other 95 per cent in 2 days. We have not been able to find the published records of these two experiments.

Several workers have made use of Umber's method, most of them for the same purpose as Umber, the study of the behavior of the gouty individual. Thus the study of the normal individual has been neglected. Dohrn,⁵⁹ during his studies of the action of atophan, injected four normal individuals, each with 500 mg. of uric acid. We cannot accept Dohrn's interpretation of the results revealed by his tables. Dohrn concludes that three of the individuals showed a quantitative recovery, while the fourth one (Table 6) excreted 63 per cent only. We must conclude that of the other three, one excreted 58 per cent only and this during the day of injection (Dohrn's Table 4); another one excreted either 39 per cent in 1 day, which seems to us the best interpretation, or 123 per cent in 5 days (Dohrn's Table 7); and the last one excreted 307 per cent in 5 days (Dohrn's Table 5). Using the same method, Griesbach,⁶⁰ in three comparatively normal individuals recorded a recovery of 22, 49, and 68 per cent, respectively, and concluded that the procedure could not be used to demonstrate the absence of uricolysis in man. To the excessive recoveries of more than 100 per cent already reviewed, can be added another two by Bass,⁶¹ where 164 and 133 per cent, respectively, were recovered.

⁵⁶ Gudzent, Wille, and Keeser, *Z. klin. Med.* 1921, xc, 147. Gudzent, F., *Berl. klin. Woch.*, 1921, lviii, 1401.

⁵⁷ Schittenhelm, A., and Harpuder, K., *Z. ges. exp. Med.*, 1922, xxvii, 14.

⁵⁸ Umber, F., and Retzlaff, K., *Verhandl. Kong. inn. Med.*, 1910, xxvii, 436.

⁵⁹ Dohrn, M., *Z. klin. Med.*, 1912, lxxiv, 445.

⁶⁰ Griesbach, W., *Biochem. Z.*, 1920, ci, 172.

⁶¹ Bass, R., *Zentr. inn. Med.*, 1913, xxxiv, 977.

During the 90's piperazine given by mouth was used in the therapeutics of gout to promote the excretion of uric acid. It seems unfortunate, therefore, that piperazine was adopted in Umber's technique. Its effect on the uric acid output is clearly demonstrated by Dohrn who, in two experiments, injected 1 gm. of piperazine intravenously into a gouty individual, and both times obtained a definite increase of the uric acid elimination. These observations become only more outstanding by the third experiment on the same individual. In this experiment the injection of 500 mg. of uric acid failed entirely to produce any increased uric acid elimination. Contrary to Dohrn's suggestion, the slightly increased diuresis (to 1,700 and 1,400 cc., respectively) after the piperazine injections does not explain the increased uric acid elimination. Bürger,⁶² working in Schittenhelm's Clinic, compared, in twelve non-gouty individuals, the recovery of injected uric acid dissolved by means of piperazine with the recovery of the same amount of uric acid dissolved in boiling water by the addition of 0.1 N sodium hydroxide according to Schade and Boden.⁶³ From the injections of the latter preparation, Bürger consistently obtained only about one-half as much as he recovered from the piperazinized uric acid injections. It is difficult to judge about Bürger's results since he evidently did not check up the amount of uric acid which might have been destroyed during the boiling of the sodium urate solution. Most likely this criticism does not influence the general trend of his results, but it makes his figures less serviceable for percentage calculations.

Other results obtained by Gudzent⁶⁴ and his coworkers may be referred to, but their figures, obtained from intravenous sodium urate injections, are so different from the results obtained by others or by ourselves that we cannot undertake to interpret them.

It is clear enough from the data recorded from the literature, that Wiechowski's theory about the indestructibility of uric acid in man has received very little support from metabolism experiments. But if that theory becomes untenable—that is if there is destruction of uric acid—there must either occur extensive destruction of allantoin as well, or else the destruction of uric acid goes in some other way, since human urine contains only insignificant traces of allantoin. From the recent papers of Biltz⁶⁴ on the chemical oxidation of uric acid one might infer that allantoin need not be the only substance produced if uric acid is oxidized within the animal body.

III.

When lithium urate solutions are taken by mouth or through a duodenal tube scarcely a trace of the ingested uric acid finds its way into the urine. Because of this remarkable fact it is not possible to study the fate of uric acid within the human

⁶² Bürger, M., *Arch. exp. Path. u. Pharmacol.*, 1920, lxxxvii, 392.

⁶³ Schade, H., and Boden, E., *Z. physiol. Chem.*, 1913, lxxxiii, 347.

⁶⁴ Biltz, H., and Schauder, H., *J. prakt. Chem.*, 1923, cvi, n. s., 108.

organism by the same sort of experiments as can be used, and have often been used, to elucidate the behavior of other equally simple substances of physiological importance, such as urea, creatinine, creatine, amino acids, or sugar. It is manifestly essential to know what does happen to uric acid within the human organism before one can accept without reservation the more comprehensive, but also more complex, story of purine metabolism as presented in the *best* modern literature. Yet it is precisely in regard to the fundamental question whether uric acid is or is not in part destroyed within the human organism that the abundant uric acid literature fails to reveal adequate investigation or convincing results.

TABLE I.

Showing the Distribution of Uric Acid in Dogs before and after Injection of Uric Acid (100 Mg. per Kilo).

Dog No.	Weight.	Uric acid per 100 gm. of tissue.						Remarks.
		Plasma.	Muscle.	Spleen.	Liver.	Brain.	Kidneys.	
	kg.	mg.	mg.	mg.	mg.	mg.	mg.	
1	7.5	0.5	1.2	2.2	1.3	1.2	1.2	Normal.
2	4.3	0.6	2.5	1.4	2.8	2.5	1.4	"
3	8.1	11.6	2.5	4.7	3.8	1.2	126.0	} 10 min. after injection of 100 mg. uric acid per kilo.
4	10.8	29.8	4.4		7.6		162.0	

The only way, probably, to settle this question is by intravenous injections of uric acid, and in this paper we shall discuss the problem on the basis of evidence furnished by injection experiments. As already intimated, we were compelled to resort to this line of work by the negative findings obtained from ordinary feeding experiments as well as from injections of lithium urate solutions through a duodenal tube. Before proceeding with a discussion of the behavior of uric acid in man we must present certain data obtained from analogous experiments with dogs, because these seem to elucidate several important aspects of the main problem.

The uric acid metabolism in the dog differs so much from the corresponding process in man that at first it seemed scarcely

worth while to include this animal in our studies. It seemed probable, however, that at least one important point, namely the immediate distribution of injected uric acid among the different tissues of the body, might be cleared up by the use of animals. The results obtained with dogs have been unexpected and not easy to interpret, and we have, therefore, been compelled to make many more experiments than we had any thought of making when we began.

In Table I are shown the results of four experiments made for the purpose of revealing the immediate distribution of injected uric acid. The first two dogs had received no uric acid and the figures obtained from these serve merely to illustrate the "uric acid" values one obtains from the tissues of ordinary normal animals. These control figures must represent uric acid in part, but they may include other products as well which also give a color with the uric acid reagent. These unknown materials are relatively abundant in the liver. The products may in part be removed by first precipitating the uric acid from the tissue extract by means of silver lactate, and in the beginning we made some of our tissue analyses by means of the silver lactate precipitation. But the figures recorded in this paper are to be interpreted as representing colorimetric estimations made directly on protein-free tissue filtrates.

The tissue filtrates were obtained in the following way.

10 gm. still warm organ were ground with 10 gm. fine sand, transferred into a liter flask with about 400 cc. boiling 0.01 *N* acetic acid, boiled for 5 minutes and cooled; the protein precipitation was then finished by means of 3 cc. 10 per cent sodium tungstate and 3 cc. $\frac{1}{4}$ *N* sulfuric acid. The mixture was made up to 500 gm. and filtered. The filtrates were either used directly for the uric acid determination or, in the case of a low uric acid content, an aliquot part was first concentrated to one-fifth. When silver lactate precipitation was used, a small amount of NaCl had to be added to the filtrate.

During the latter part of this work the filtrates have been obtained in a simpler and even more satisfactory way. 5 gm. still warm organ were well ground with 5 gm. fine sand and 5 cc. 10 per cent sodium tungstate. The mixture was transferred into a 500 cc. flask, made up to 250 gm., thoroughly shaken and the precipitation finished by means of 5 cc. $\frac{1}{4}$ *N* sulfuric acid. Filtration. According to the uric acid concentrations expected in the organ, the degree of dilution has sometimes been modified, the mixture having been made up to 125 or to 500 gm.

Even for the blood plasma the dilutions have been varied according to the expected concentrations. Thus plasma dilutions varying from 1:5 up to 1:100 have been used.

All the uric acid determinations were made by means of the improved uric acid reagent described in the following paper and by the help of a sodium cyanide which alone gave absolutely no color with the reagent.

Animals whose blood contains at the most only a small fraction of a milligram of uric acid per 100 cc. of blood and whose urine is nearly free from uric acid might, nevertheless, carry uric acid in the tissues, including the muscles. There is no evidence showing that such animals do not produce endogenous uric acid. On the contrary, all the available evidence points to production followed by destruction, and is so interpreted. On the basis of this point of view the muscles of the dog should contain uric acid as surely as the muscles of man, though the quantity might be smaller.

From the preliminary analyses recorded in Table I it is clear, first, that the kidneys have a special power to abstract uric acid from the circulating blood, and, second, that it is doubtful whether circulating uric acid can enter any of the other tissues. Since the excised tissues always contain some blood, absolutely negative values for tissue uric acid are practically unobtainable when the blood contains uric acid. To us it seems untenable to assume that any uric acid can enter other tissues than the kidneys when the amount found is so small that it can be due to the unknown preexisting reducing materials plus the uric acid of the blood. Substances which can enter the tissues, urea, creatine, and creatinine, do so with the utmost speed up to the point of approximate equilibrium between the concentration in the blood and in the tissues. There is no reason for assuming that forces other than those of diffusion are responsible for the distribution of those other substances. Complete blocking of such diffusion of the injected urate seems more reasonable than nearly complete blocking, since urates diffuse rapidly through ordinary membranes, including dead animal membranes. The point here raised is of far reaching theoretical importance, not only for a correct interpretation of the subsequent experiments recorded in this section, but also for an understanding of the distributions obtained with human sub-

jects. We, therefore, want to make it perfectly clear that our conclusion that the uric acid does not enter such tissues as the muscles represents, to a certain extent, interpretation rather than complete experimental proof. It may be argued that the distribution of uric acid to the tissues might be nearly as rapid as the distribution of urea or creatinine, since this fact could be masked by a correspondingly rapid destruction. One effect of such a destructive process, however, should be to hasten greatly the diffusion of the uric acid into the tissues—yet we are perfectly certain that the distribution process for uric acid is of a relatively low order. According to present day accepted teachings, there is no teleological reason why uric acid should diffuse into muscular tissues since “the uric acid is destroyed in the liver.”

This view, in so far as it is correct, would serve to confirm the validity of our conclusion that uric acid is not distributed to the muscular tissues. But our analyses also indicate that there is no distribution of injected uric acid to the liver. This special aspect of uric acid distribution (and destruction) can be better discussed in connection with some of our later experiments. For the present, therefore, we only emphasize that at the most there can be but an insignificant distribution of injected uric acid to tissues other than the kidneys.

In the absence of a general distribution of injected uric acid it is obvious that the rate of destruction can be followed by means of blood analyses very much better than would be the case if the uric acid were freely distributed to all the tissues in the body. This destruction is so rapid that the excretion by the kidneys can be disregarded, especially since the kidneys, as a matter of fact, excrete only traces of uric acid in response to injections of 100 mg. per kilo of body weight. The following experiment may be cited to illustrate the apparent speed of uric acid destruction in the dog. 100 mg. per kilo were injected in 2 minutes. The first blood was taken 30 seconds later. It contained 141 mg. of uric acid per 100 cc. of plasma. A second sample, taken 24 minutes later, gave a plasma value of only 40.6 mg. 91.4 mg. per 100 cc. of plasma had thus disappeared in the course of 24 minutes. From other experiments we have reason to believe that none of the disappearance can be ascribed

to absorption by the kidneys, for they become filled up even before the injection is finished. Notwithstanding the extraordinarily high uric acid level found in the first blood, the greater part must have been destroyed before that blood was taken—in other words, during the injection. It is, therefore, not possible

TABLE II.

Showing Rate of Disappearance (Destruction) of Uric Acid in the Dog.

						Uric acid per 100 cc. plasma.
						mg.
1.	100 mg.	of uric acid	per kilo	injected	in 4 min.	
2.	1st blood	immediately	after	injection	72.8
3.	2nd	"	3 min.	after	injection	30.6
4.	3rd	"	4	"	"	25.4
5.	4th	"	16	"	"	12.1
6.	5th	"	17	"	"	10.8

TABLE III.

Showing Rate of Uric Acid Destruction in the Dog, As Measured by Disappearance from Blood.

Dog A was injected with 200 mg., Dog B with 100 mg. of uric acid per kilo.

Time after injection of uric acid.	Uric acid per 100 cc. plasma.	
	Dog A.	Dog B.
	mg.	mg.
2 min., 15 sec.	80	
2 " 57 "		37.6
3 " 43 "	56	
4 " 17 "		24.6
5 "	39	
5 " 24 sec.	26.8	
5 " 41 "		16.4
11 " 13 "		7.0
11 " 23 "	19.8	
15 " 57 "		5.6
16 " 50 "		1.8

to follow by blood analysis the initial extremely rapid destruction of uric acid; but we can follow the course of the process after the mixing within the circulating blood has been completed. At one time it seemed as if the rate of uric acid destruction, as indicated by its disappearance from the blood, was so regular that the process

might be expressed in the form of a simple mathematical formula, and we have, therefore, made a considerable number of carefully timed series of blood analyses after injecting uric acid. As the rate of uric acid disappearance has not quite reached the expected regularity we confine ourselves to the presentation of the figures recorded in Tables II and III.

From the figures recorded in these tables it is clear that we are here dealing with a process so rapid and at the same time so definite that one might fairly expect to be able to work out

TABLE IV.

Showing that Muscle of Dog Does Not Absorb and Does Not Destroy Uric Acid.

	Found in 100 cc. plasma.	
	Non-protein N.	Uric acid.
	mg.	mg.
1. Femoral veins and arteries exposed.		
2. 100 mg. of uric per kilo injected in 2½ min.		
3. 1 min. after injection took blood from left femoral vein.....	54.0	59.6
3a. 1 min. later took blood from left femoral artery...	52.8	51.2
4. 8½ " after injection took blood from right femoral vein.....	43.5	21.6
4a. 45 sec. later took blood from right femoral artery...	41.4	17.8
		Uric acid per 100 gm.
		mg.
5. 3 min. after injection removed left gracilis.....		3.8
6. 18 " " " " kidneys.....		116.0

some of the essential factors involved. It ought to be possible, at least, to determine where this rapid destruction takes place. The experiments recorded in this section are intended to elucidate this primary question.

One of our early experiments bearing on the problem of the localization of the uric acid destruction is recorded in Table IV. The dog used weighed 20.5 kilos, and received, through the vein of the right fore leg, 2,050 mg. of uric acid in about 1 per cent solution. Before the injection the dog had been anesthetized, and the femoral veins and arteries of both hind legs were exposed.

The figures of Table IV obviously lend no support to the hypothesis that the uric acid might be speedily absorbed and as speedily destroyed by muscles. The gracilis muscle was removed 3 minutes after the injection of the uric acid, and at that time was being perfused with blood containing from 51 to 59 mg. of uric acid per 100 cc. of plasma, yet by analysis it gave us only 3.8 mg. per 100 gm. of tissue. The analyses of each pair of venous and arterial bloods show that the venous blood is distinctly richer in uric acid than the arterial blood—

TABLE V.

Showing that Muscle of the Dog Does Not Absorb or Destroy Uric Acid; Also that the Kidney First Absorbs, and Later Gives Off, Uric Acid.

	Uric acid in 100 cc. plasma.	
	Venous.	Arterial.
	mg.	mg.
1. Femoral and renal veins and arteries exposed.		
2. 100 mg. of uric acid per kilo injected in 2½ min.		
3. 75 sec. after injection took blood simultaneously from left femoral vein and artery.....	62.4	66.0
4. 8 min. after injection took blood simultaneously from right femoral vein and artery.....	25.8	26.2
5. 14 min. after injection took blood simultaneously from left renal vein and artery.....	20.4	14.4
		Uric acid per 100 gm.
		mg.
6. 5 min. after injection removed left gracilis.....		5.2
7. 10 " " " " right ".....		4.5
8. 15 " " " " left kidney.....		157.0

59.6 versus 51.2 mg. in the first pair, and 21.6 versus 17.8 in the other. The venous bloods were taken first in this experiment, because one is more apt to encounter some delay in drawing venous than in the drawing of arterial blood. The higher uric acid levels in the venous bloods obviously reflect merely the fact that they were drawn first, since the general level of uric acid in the blood was constantly and rapidly falling. The values obtained showed us that conclusive results can be obtained only by taking strictly simultaneously the arterial and venous

bloods corresponding to the tissue whose function is to be examined.

The next experiment which we wish to put on record is represented in Table V. In this case we exposed, as before, the femoral veins and arteries of the two hind legs, and, in addition, the renal artery and vein of the left kidney. The dog weighed 12.6 kilos, and received through the vein of the right front leg 1,260 mg. of uric acid, in the form of a freshly prepared lithium urate solution.

In practically all of our injection experiments we have determined the non-protein nitrogen as well as the uric acid. The non-protein nitrogen figures belonging to Table V are as follows: (3) 42 and 42.6 mg., (4) 30.9 and 31.2 mg., and (5) 35.7 and 31.5 mg.

The first pair of uric acid figures recorded in Table V would seem to indicate that a definite loss, 3.6 mg., had occurred while the blood passed through the muscles of the left hind leg. But in this case we did not attain strictly identical speed of collection. The arterial blood at first rushed into the collection pipette much faster than the corresponding collection of venous blood. This small difference seemed at the time a sufficient explanation, because these bloods were taken only 75 seconds after a fairly rapid injection and the destruction was unquestionably proceeding excessively fast.

The figures obtained for the second pair of bloods, 25.8 and 26.2 mg., on the other hand, show clearly that the continuous disappearance of circulating uric acid cannot be ascribed to the passage through muscle.

In the course of our efforts to prove by further experiments that the passage of the blood through muscles involves no loss of uric acid at a period when we know that there is a very rapid loss somewhere, we began to get results which were entirely different from those recorded in Table V. The arterial blood would sometimes contain up to 10 or 14 mg. more uric acid than the venous blood under conditions of perfectly simultaneous removal. At other times the figures would again be quite identical. The reason for these discordant results was that we had underestimated the time required to secure complete mixing of the injected uric acid within the circulating blood. It apparently

requires about three complete circulations, 3 minutes counting from the end of an injection into the vein of a front leg of a dog, before all the blood is thoroughly mixed with the urate solution. If the bloods are taken much earlier than that the venous sample is apt to contain some blood which has lingered in the capillaries long enough to escape complete mixing. The first pair of bloods recorded in Table V are of this order.

The following figures from three different dogs may be cited to show that the disappearance of uric acid is not due to the passage through the muscles (Table VI).

The results obtained from the kidneys, in Table V, are both surprising and illuminating. The destruction of uric acid must take place where the uric acid is; and from this point of view

TABLE VI.

Showing that Venous and Arterial Blood Are Identical in Uric Acid Content.

Dog No.	Time from end of injection to taking of bloods.	Uric acid per 100 cc. plasma.	
		Arterial.	Venous.
		mg.	mg.
1	5 min. (200 mg. uric acid per kilo)....	66.8	65.6
2	3 " (100 " " " " " ")....	28.2	28.4
3	3½ " (100 " " " " " ")....	41.2	44.4

there was reason to expect that one important seat of uric acid destruction should be the kidneys—though it might also be possible that excessive uric acid concentration in this organ is only a preliminary step to its excretion with the urine. A third interpretation is, however, clearly indicated by the data obtained.

The kidney removed 15 minutes after the injection contained 157 mg. of uric acid per 100 gm. The arterial blood passing into this kidney 1 minute earlier contained 14.4 mg. per 100 cc. of plasma, while the venous blood coming away from the organ contained 20.4 mg. These figures prove that the kidney was rapidly giving off uric acid to the circulating blood.

From these clear-cut data it is evident that the kidneys first take up uric acid from the circulating blood and a little later give it up to the same medium. There is no reason to assume that the kidneys also serve as a special seat for uric acid destruction.

In saying this we do not imply that there is no destruction within the kidney tissue. But the special or exclusive seat of uric acid destruction, if there be any in the dog, is not located in the kidneys. There is some evidence, the high non-protein nitrogen of the blood which has passed through the kidneys, which might be interpreted as signifying an extensive breakdown of uric acid within the kidney tissue, but the real significance of this nitrogen is still decidedly obscure. (Compare the corresponding figures in Table VIII.)

TABLE VII.
*Showing Temporary Retention and No Specialized Destruction of
Uric Acid by the Kidneys.*

	Uric acid per 100 cc. plasma.	
	Arterial.	Venous.
	<i>mg.</i>	<i>mg.</i>
1. Renal arteries and veins exposed.		
2. 100 mg. of uric acid per kilo injected in 4 min.		
3. 2 min. after injection took blood simultaneously from left renal artery and vein.....	20.8	20.2
4. 4 min. after injection took blood from right renal artery.....	6.3	
4a. 1½ min. later took blood from right renal vein....		13.4
		Uric acid per 100 gm. tissue.
		<i>mg.</i>
5. 3 min. after injection removed left kidney.....		75.0
6. 7 " " " " right "		45.6

Further evidence showing that the kidneys serve as a temporary receptacle for a part of the injected uric acid is given in Table VII. The dog used in this experiment weighed 10 kilos, and received 1,000 mg. of uric acid. In order to facilitate ready inspection we have omitted the non-protein nitrogen figures from the table. In the first pair of arterial and venous bloods we obtained 42.3 and 45 mg., respectively, of non-protein nitrogen, while in the second pair, the corresponding figures are 44.4 and 58.8 mg. of non-protein nitrogen. Here we have an increase of over 14 mg. in the non-protein nitrogen of the blood, acquired

while passing through the right kidney, less than 3 of which are represented by uric acid. But there was more uric acid in the first (left) kidney than in the right and there must have been more rapid destruction of uric acid within the dog when we collected the blood from the left kidney, yet there the accumulation of non-protein nitrogen by the venous blood was less than 3 mg.

The uric acid values of the blood and the kidneys seem to us particularly significant. The substantial identity of the uric acid figures (20.8 and 20.2 mg.) obtained from the blood plasma going in and out of the left kidney shows that the kidney at this time neither adds nor abstracts uric acid in quantities sufficiently pronounced to be revealed by the blood analysis. That the kidney had previously taken up uric acid is proved by the 75 mg. per 100 gm. actually found in that kidney. In striking contrast to the uric acid equilibrium in the left kidney we see that the right kidney, examined 2 to 3½ minutes later, is rapidly giving off uric acid to the passing blood. The arterial blood plasma entering this kidney has only 6.3 mg., while the venous plasma coming out has 13.4 mg. of uric acid. The difference between these two analyses would probably have been greater still if the arterial and venous bloods had been taken simultaneously as was done in the case of the left kidney, for during the 1½ minutes which elapsed between the collection of the arterial and the venous bloods the general level of circulating uric acid had, of course, been materially reduced. During a period of 2 minutes immediately preceding (the interval between the collection of the two samples of arterial blood) the uric acid had fallen from 20.8 to 6.3 mg. Corresponding to the speedy transfer of uric acid from the right kidney to the passing blood we see that this kidney contained only 45.6 mg. of uric acid per 100 gm. as against the 75 mg. found in the left.

It seems indeed remarkable, not to say incredible, that such strikingly different figures for the uric acid traffic between blood and kidneys should be obtained in the same animal and with only 2 minutes elapsing between the starting points for each set of analyses. But 2 minutes is a long period, in relation to a process so rapid as the destruction of uric acid in the dog. It is quite possible that the removal of the left kidney has had some influence on the processes going on in the remaining right kidney.

The influx of allantoin and other decomposition products of uric acid, already alluded to, may have promoted to a considerable extent the exit of the uric acid from the remaining kidney, although the results recorded in Table V show that the removal of one kidney has not been the sole or main cause of the release of uric acid by the remaining kidney, since in Experiment 5 both kidneys were left undisturbed.

The customary method of showing whether a given organ is responsible for a given result is to remove the organ. While

TABLE VIII.

Showing Storage and Subsequent Release of Uric Acid and Non-Protein N by the Kidneys.

	Per 100 cc. plasma.	
	Non-protein N.	Uric acid.
	mg.	mg.
1. Renal arteries and veins of kidneys exposed.		
2. Injection of 100 mg. of uric acid per kilo in 6 min.		
3. 2½ min. after injection took blood from left renal vein.....	70.2	36.0
4. 14 min. after injection took blood from right renal artery.....	72.6	12.2
4a. 30 sec. later took blood from right renal vein.....	100.2	27.8
		Uric acid per 100 gm.
		mg.
5. 6 min. after injection removed left kidney.....		160.0
6. 15 " " " " right "		94.0

we entertained no doubt about the fact that the kidneys of the dog have little or nothing to do with the observed destruction of injected uric acid, it was thought best to make one or two experiments bearing on this point. The results of the first experiment are recorded in Table IX.

This experiment was made before we had become thoroughly familiar with the fact that most of the administered uric acid is destroyed almost as fast as it is injected. In Table IX the first blood was taken 7 minutes after a 5 minute injection. The finding of only 26.2 mg. of uric acid in the plasma of this blood, in conjunction with the demonstrated lack of general distribu-

tion to tissues, shows that by far the greater part of the given uric acid had already vanished. The subsequent samples of blood give figures which show clearly enough the continuous but steadily diminishing rate of uric acid destruction, until about 2 hours after the injection, when the blood plasma has only 1 mg.—that is a trifle more than might be found in any dog's blood. The appended tissue analyses complete the picture. 2 hours after the injection of 100 mg. of uric acid per kilo of body weight, there was no uric acid left in this nephrectomized dog.

TABLE IX.

Showing that the Kidney Is Not the Seat of Uric Acid Destruction.

	Per 100 cc. plasma.	
	Non-protein N.	Uric acid.
	mg.	mg.
1. Both kidneys removed.		
2. Injected 100 mg. of uric acid per kilo in 5 min.		
3. 7 min. after injection took blood	30.6	26.2
4. 23 " " " " "	30.6	11.2
5. 44 " " " " "	30.0	5.9
6. 79 " " " " "	30.6	2.4
7. 2 hrs. " " " " "	31.5	1.0
		Uric acid per 100 gm.
		mg.
8. 2½ " " " removed:		
(a) Muscle		1.4
(b) Pancreas		1.4
(c) Liver		1.4
(d) Mucosa of jejunum		4.1(?)

In a second experiment, made 2 days after the one recorded in Table IX, we again injected 100 mg. of uric acid per kilo of body weight, after having excised the kidneys. This injection lasted 6 minutes, and the first blood was taken 4 minutes later. This contained 36 mg. of uric acid per 100 cc. of whole blood, or about 55 mg. per 100 cc. of plasma. 5 minutes later a second sample gave 13 mg., corresponding to about 20 mg. per 100 cc. of plasma. Immediately after the taking of the second blood, the animal was killed and certain tissues removed and analyzed.

for uric acid. The following figures were obtained: muscle 3.4 mg., intestine 3.5 mg., pancreas 10.4 mg., and liver 4.2 mg. These analyses supplement in a measure the results shown in Table IX, and leave no room for doubt about the speedy destruction of uric acid, without any demonstrable distribution to tissues, in dogs without kidneys.

The experiments recorded in the preceding tables show that the rapid destruction of uric acid in the dog cannot be ascribed to such general and bulky tissues as the muscles, or to the kidneys. Our distribution figures have also failed to furnish any support for the hypothesis that the chief seat of uric acid destruction may be localized in the liver. When it comes to the liver it is imperative, however, to consider again most carefully whether the apparent lack of absorption from the blood has not been masked by the special, localized destruction of uric acid, since all pertinent investigations point unmistakably to the liver as the chief seat of the uric acid-destroying process. We would recall in this connection not only the numerous uricase studies referred to in our review of the literature, but particularly Mann's liver extirpation experiments, which show that the removal of this organ promptly leads to a rapid accumulation of uric acid in the blood of dogs.

The Eck fistula operation on the dog used in Experiment 10 was made for us by a surgeon, Dr. E. C. Cutler. The success of the operation was verified by autopsy. The dog recovered well from the operation and was in good condition at the time of our experiment. The animal weighed 12 kilos, and received 1,200 mg. of uric acid. This dog, like all others permitted to survive the uric acid injection, became quite sick, but recovered seemingly completely in a few days. On the 1st day after the injection the dog was still very dull and apathetic and took very little food. The urine obtained by catheterization on the 2nd day contained albumin (trace), many blood cells, and some epithelial and large granular casts.

The uric acid figures recorded in Table X show that the greatly diminished circulation through the liver produced by the Eck fistula was without demonstrable effect on the uric acid destruction. Within 4 minutes after a fairly rapid injection of 100 mg. of uric acid per kilo of body weight, the greater part had been de-

stroyed, since the circulating plasma contained only 34.4 mg. per 100 cc. This observation certainly does not indicate that the liver is the *seat* of uric acid destruction. The uric acid value obtained 2 hours after the injection, 2.9 mg., is a trifle larger than the corresponding value obtained after removal of the kidneys (Table IX), but the difference is quite insignificant. In passing we would call attention to the high non-protein nitrogen found in the plasma of this Eck fistula dog, 26 hours after the uric acid injection. Such nitrogen accumulations are always obtained in dogs allowed to survive the uric acid injections.

TABLE X.
Showing Destruction of Uric Acid in an Eck Fistula Dog.

	Per 100 cc. plasma.	
	Non-protein N.	Uric acid.
	mg.	mg.
1. Dog catheterized. Urine (normal) contained 73 mg. of uric acid per 100 cc.		
2. 100 mg. of uric acid per kilo injected in 3 min.		
3. Venous blood taken 4 min. after injection.....	15.6	34.4
4. " " " 26 " " "	23.7	16.4
5. " " " 64 " " "	23.1	8.5
6. " " " 2 hrs. " " "	28.2	2.9
7. " " " 26 " " "	73.2	None.
8. Dog catheterized 33 min. after the injection. The urine (13 cc.) contained 23 mg. uric acid.		

We interpret the data obtained from this Eck fistula dog as indicating that the liver is not the seat of the uric acid destruction. This interpretation is based entirely on the observed speed of uric acid disappearance from the blood. The surplus power of a given organ to accomplish a given result as, for example, the prevention of experimental diabetes by a small fraction of the pancreas, or the adequate excretion by only half a kidney, has no definite application in this case, because we are comparing maximum accomplishments, and one essential factor in a localized destruction of circulating uric acid must necessarily be the frequency with which all the uric acid is made to pass through that particular locality, in this case the liver. The Eck fistula liver has had less than 10 per cent as much opportunity to abstract

and destroy the circulating uric acid as have the livers of normal dogs, yet the speed of destruction is substantially the same.

It is conceivable, though highly improbable, that the uric acid is normally destroyed in the liver, but that through some obscure vicarious action the uric acid destruction is transferred to other tissues when the liver is unable to do the work, as in our Eck fistula dogs. We, therefore, made a couple of experiments to determine what happens to the uric acid when made to pass directly through the portal circulation. Our first attempt was not very successful because, when the needle connecting with

TABLE XI.

Showing that Preliminary Passage of Uric Acid through the Liver Does Not Alter the Distribution or the Rate of Destruction.

		Uric acid per 100 cc. plasma.
		mg.
1.	Right kidney mobilized.	
2.	100 mg. of uric acid per kilo injected through mesenteric vein in 5 min.	
3.	4 min. after injection took blood from heart.....	29.6
4.	16 " " " " " " " "	8.0
		Uric acid per 100 gm.
		mg.
5.	2 " " " removed right kidney.....	66.5
6.	6½ " " " left "	38.0
7.	18 " " " section of liver.....	1.4

the uric acid reservoir was inserted in the large portal vein, some of the solution (and blood) leaked out around the needle. The injection was slow, lasting 11 minutes. 3 minutes after the injection, one lobe of the liver was removed and was found to contain only 3.6 mg. of uric acid, while the left kidney removed 3 minutes later contained 72 mg.

In the next experiment the uric acid solution was introduced through a branch of the superior mesenteric vein and no difficulty as to leakage was encountered. The results obtained are shown in Table XI. The dog weighed 12.6 kilos, and received 1,260 mg. of uric acid. This dog happened to have chronic nephritis. The probable existence of nephritis was inferred from the gross

appearance of the kidneys, and was confirmed by microscopic examination of some mounted sections (by B-d). Because of the large amount of connective tissue replacing normal kidney cells, the abstraction of uric acid from the blood by these kidneys could be and undoubtedly was materially diminished. Since the kidney is not the seat of uric acid destruction the lessened absorption by that organ does not alter the significance of the results obtained.

The figures recorded in Table XI clearly do not support the assumption that the liver is the seat of uric acid destruction. 4 minutes after the injection we find 29.6 mg. in the plasma, while in the Eck fistula dog (Table X), the 4 minute plasma contained 34.4 mg., and in Table II the 4 minute plasma contained 25.4 mg. If there had been any difference, that fact should have been revealed by these first samples of blood, because the liver in Experiment 11 must have had at least twenty times as much uric acid at its disposal as the liver of the Eck fistula dog. We, therefore, definitely conclude that the liver is not the special seat of uric acid destruction in the dog.

One other experiment showing that the liver is not the seat of the metabolic destruction of uric acid in the dog may be cited here. The main purpose of this experiment was to determine whether uric acid is absorbed from the intestinal tract. 315 mg. of uric acid were dissolved by the help of 150 mg. of lithium carbonate in about 50 cc. of warm water and injected into an isolated loop of the jejunum about 30 cm. long. This dog weighed 10.2 kilos, so that we were here dealing with a very small amount of uric acid in comparison with the quantities used in the direct injection experiments, and, in addition, the uric acid was passing through the liver at a comparatively very slow rate. 289 mg. of uric acid, or 28 mg. per kilo of body weight, were absorbed in 51 minutes. In an experiment of this sort it is out of the question to expect the accumulation of much uric acid in the blood. When 100 mg. per kilo are injected, at least 70 per cent is destroyed within the first 10 minutes, but toward the end, when only 1 or 2 mg. of circulating uric acid per 100 cc. of plasma are left, the destructive process becomes distinctly slow. Whether we should or should not find any uric acid in the general circulation in response to the relatively slow absorption from an intestinal

loop was, therefore, necessarily uncertain. The uric acid contents of the peripheral blood plasma rose from a very slight trace (probably less than 0.3 mg.) to 1.1 mg. Small as this increase is, it is unmistakable, and proves that the uric acid-destroying power of the liver is very limited indeed. It is, therefore, quite impossible to localize in the liver, a uric acid destruction which in speed approaches that of a chemical titration and which occurs during a rapid injection of uric acid into the blood stream.

TABLE XII.

Showing that Uric Acid Is Absorbed from the Intestine and Passes through the Liver of Dogs.

	Uric acid per 100 cc. plasma.
	mg.
1. 315 mg. of uric acid injected into isolated intestinal loop.	
2. Peripheral blood taken just before uric acid injection.....	Trace.
3. Blood from mesenteric vein taken 20 min. after injection.....	2.2
4. Peripheral blood taken 26 min. after injection.....	0.7
5. Blood from mesenteric vein taken 28 min. after injection.....	1.9
6. Blood from mesenteric vein taken 43 min. after injection.....	2.1
7. Blood from heart taken 48 min. after injection.	1.1
8. Uric acid left in intestinal loop after 51 min., 26 mg.....	Absorbed 92 per cent.

Having proved, at least to our own satisfaction, that uric acid is not destroyed in the muscles, or, to a demonstrable extent, in the kidneys, or in the liver of the dog, we are practically forced to conclude that the seat of uric acid destruction, in this animal at least, is in fact the circulating blood. The destruction of the uric acid shown by analysis of blood samples taken at different intervals proceeds with such great velocity that if the destructive process were localized in some particular organ it would have to represent such an extensive reduction of the uric-acid content of the passing blood that it is impossible for us to believe that we might not have found it.

We recognize that in ascribing the locus of the uric acid destruction to the circulating blood we lose the support of the reasonableness and probability inherent in the well established view that the chemical transformations occurring in animal organisms are produced within the living cells—a view which, since Virchow's time, has grown stronger and stronger with each succeeding decade. There are, however, well established exceptions as, for example, in the field of digestion. It is also to be noted that during the past 20 years or more the concept of specific endocellular, as well as exocellular, enzymes has been steadily gaining ground, and this expanding enzyme concept has necessarily encroached on the concept of direct protoplasmic activity as the cause of chemical transformations. All recent investigations on the destruction of uric acid by the different tissues of the body represent studies of the distribution of "uricolytic enzymes." While the authors accept the endocellular character of these enzymes, they also obtain more or less active "tissue extracts." The speed of uric acid destruction which has been obtained by means of uricolytic enzyme preparations can scarcely be compared with the extraordinary rate of uric acid destruction which takes place during and immediately following the injection of uric acid into the blood of a living dog.

It is not now necessary to express an opinion as to whether the uric acid destruction in the blood can be interpreted as a consequence of a corresponding influx of the uricolytic enzymes found by Wiechowski, Jones, and others, including Schittenhelm. There is not necessarily any inconsistency between their findings and ours, since their uricolytic enzymes might represent remnants of some "internal secretion" abundantly available in the living animal. On the other hand, the uric acid destruction in the blood need not be produced by an agency so specific in character as the uricolytic enzymes are supposed to be. Uric acid in solution is a comparatively unstable substance and is particularly easily oxidized. Just as no one has considered it necessary to ascribe the rapid disappearance of adrenalin from blood to the presence of some highly specific enzyme, so the disappearance of uric acid may be due in large part to oxidative decompositions of a more general and less closely regulated character.

The transformation of a large part of the uric acid into allantoin in the course of such oxidations would follow as a matter of course from the neutral or faintly alkaline reaction of blood, but at present it is scarcely justifiable to assume that allantoin is the only product formed. One reason why it is difficult or impossible to ascribe the uric acid destruction in the circulating blood to highly specific uricolytic enzymes is the fact that the destruction process stops as soon as the blood is withdrawn from the circulation within the living animal. There is perhaps little reason to assume that ordinary dog blood should contain appreciable quantities of the hypothetical uricolytic enzymes since

TABLE XIII.

Showing that There Is No Destruction of Uric Acid in Blood after Removal from the Circulation.

	Uric acid per 100 cc.	
	Whole blood.	Plasma.
	mg.	mg.
1. 100 mg. of uric acid per kilo injected in 2 min.		
2. Dog bled immediately (carotid artery), time 6 min.		
3. 35 min. after bleeding.....	22.2	35.2
4. 1½ hrs. " "	22.0	35.6
5. 2¼ " " "	22.8	35.2
6. 3¼ " " "	22.4	35.0
7. 21 " " "	22.4	35.6

ordinarily there is very little circulating uric acid to be destroyed. But if blood is drawn immediately after an intravenous injection of uric acid and an extremely active process of uric acid destruction has been started, then the uricolytic enzymes ought to be revealed—if they are ever there.

The figures recorded in Table XIII show that the destruction of uric acid in the circulating blood must be due to some agency or condition which is lost when the blood is removed from the living animal. The destructive agency which thus loses its activity the instant the blood is removed from the animal cannot be an enzyme. The figures of Table XIII do not show whether some uric acid destruction might not have occurred during the first 2 or 3 minutes after taking the blood, as might be the case if

the active agency were some labile oxidizing agent which is itself used up in the process. We, therefore, repeated the experiment with another animal. In this case, blood was taken from the carotid 1 minute after a rapid uric acid injection. This blood was worked up as whole blood, first within a few seconds and then at the end of 1 hour. The values obtained were exactly the same. The uric acid destruction certainly stops the moment the blood is removed from the animal.

Our conclusion that the uric acid is destroyed within the circulating blood does not exclude that some essential factor may be contributed by one or another tissue, or by all the tissues. Some agency for activating oxygen within the blood is clearly necessary, and this agency is presumably identical with the "uricolytic enzyme," "uricase" of earlier investigators. The essential point is that we ascribe the destruction of uric acid to the locality where the uric acid is—the blood, and perhaps also the kidneys—rather than to the locality which produces the unknown oxygen activator. The latter may be only some reducing substance which is used up practically as fast as it is poured into the blood. It may be pertinent to remark here that we are quite prepared to admit that the liver may be the source, or the chief source of the unknown oxidizing agent—but in this investigation we have been chiefly attempting to determine *where* the destruction occurs.

The abrupt and complete stoppage of the uric acid-destroying process has, of course, led us to question seriously the validity of our conclusion that the uric acid is destroyed within the circulating blood. But the only other possible alternative is that it is destroyed everywhere within the tissues. The hind leg of an animal represents, however, much tissue material, and we cannot reconcile the observed speed of destruction with our inability to prove that the passage through the tissues of the hind leg results in any loss, especially since there has been no demonstrable diffusion of uric acid into the muscular tissues. It is unfortunate that no precise data are available as to the time required for the blood to pass through a section like the hind leg, but a considerable fraction of the total circulation time, 1 to 1½ minutes, must be represented by the passage through such an area as the muscles in question. 10 to 15 seconds should be

ample to prove the occurrence of the uric acid destruction. The time required for the passage of the blood through the kidneys has been given as 17 seconds, and we have seen how ample a single passage of the blood has been to show the removal of the uric acid from that organ. That removal is scarcely any more rapid than is the destruction, 2 to 3 minutes after a rapid injection.

Before leaving the subject of the behavior of uric acid in dogs, we would present a few additional rather instructive experiments.

TABLE XIV.

Showing that Uric Acid Accumulated in the Kidney Need Not Be Eliminated with the Urine.

	Uric acid.
	mg.
1. Left kidney mobilized. Right ureter severed at brim of pelvis and cannula inserted.	
2. 100 mg. of uric acid per kilo injected in 6 min.	
3. Uric acid per 100 cc. plasma after 6 min.....	23.0
4. " " " 100 " " " 2 hrs.....	1.9
5. " " " in 1st urine (5 cc.) passed in 19 min.....	12.7
6. " " " 2nd " " " 2 hrs.....	None.
	Uric acid per 100 gm.
	mg.
7. Left kidney removed 10 min after injection.....	128
8. Right " " 2½ hrs. " "	3.8
9. Albumin present in the 2nd, but not in the 1st, urine.	

We cannot doubt that the destruction of uric acid is due to oxidation. Since the removal of the pancreas profoundly alters the oxidation processes with reference to carbohydrates it seemed worth while to determine whether the removal of this organ has any demonstrable effect on the destruction of uric acid. Into a dog whose pancreas had been removed about 5 hours before and whose blood sugar had risen to 300 mg. we injected the usual 100 mg. of uric acid per kilo. The first blood taken 5 minutes after the injection contained 40.8 mg. 10 minutes later the figure was 23 mg. In another 15 minutes it had fallen to 8.20; and 2 hours after the injection the uric acid had fallen

to the usual low normal level of dog plasma. The pancreas, therefore, has nothing to do with the oxidation of uric acid.

We have said very little about the elimination of a part of the injected uric acid with the urine, but have left the reader to infer that the elimination is so small as to be of no significance. We have made many observations bearing on the uric acid elimination, but Table XIV must suffice. From this experiment it may be seen that the left kidney had 128 mg. of uric acid per 100 gm. of tissue. There is no reason to assume that the other kidney had less, yet the urine collected through a cannula from this other kidney gave us a total of 12.7 mg. of uric acid. This uric acid was excreted some time during the first 19 minutes of collection. Thereafter no uric acid could be found. The uric acid excretion in response to the injection of 100 mg. of uric acid per kilo of body weight is therefore limited to the few milligrams that may come out during the first few minutes.

That the kidney tissue and the kidney function are put under very heavy strain by intravenous uric acid injections is clear enough even without microscopic or chemical examinations. Immediately after the injection, the kidneys swell up to nearly twice their normal size. They become round and hard and the surface becomes very shiny—all indicating extreme edema. None of the other organs or tissues have shown any similar change. The swollen condition of the kidneys is greatest a few minutes after the injection, and since this swelling subsides fairly rapidly it is probable that this visible effect on the kidneys is due to the uric acid itself, rather than to any decomposition product of uric acid. Transient as are the visible effects of the uric acid on the kidneys, these might well be sufficient to produce considerable and less transient effects on the function, and it must be obvious from our results that intravenous administrations of a substance which rushes exclusively, or at least predominantly, to one small vital organ such as the kidney, are not without danger, even though the substance be a normal waste product.

In Table XV are presented some figures which tend to prove that the kidney function is temporarily impaired by the injection of uric acid. The dog of this experiment weighed 12 kilos, and received only 720 mg. of uric acid, 60 mg. per kilo of body

weight. 3 hours after the injection the uric acid of the plasma had fallen to the ordinary trace of dog plasma, and we have every reason to believe that all of the administered uric acid had disappeared. In those 3 hours the non-protein nitrogen had risen from 46.2 to 79.8 mg. and the urea nitrogen from 28.8 to 50.8 mg. Any visible rise in the waste products of the blood which can fairly be ascribed to inadequate kidney function signifies a very pronounced disturbance of that function, since the large margin of safety or of surplus capacity of the organ must also be wiped out, at least in large part, before the kidney begins

TABLE XV.
Showing Impaired Kidney Function with Recovery after Injection of Uric Acid.

	Per 100 cc. plasma.		
	Non-protein N.	Urea N.	Uric acid.
	mg.	mg.	mg.
1. Injected 60 mg. of uric acid per kilo in 3 min.			
2. Blood before injection.....	46.2	28.8	0.6
3. " 5 min. after injection.....	58.8	27.9	11.7
4. " 10 " " "	60.6	27.6	8.2
5. " 15 " " "	57.0	31.8	6.5
6. " 25 " " "	58.8	33.0	4.2
7. " 50 " " "	60.6	33.9	3.0
8. " 3 hrs. " "	79.8	50.8	Trace.
9. " 22 " " "	78.0	59.9	"
10. " 2 days " "	51.6		
11. " 5 " " "	30.6		

to become demonstrably incapacitated. The subsequent figures in Table XV indicate that it took this dog at least 2 days to recover an adequate kidney function.

From the fact that the intravenous injection of 60 to 100 mg. of uric acid per kilo of body weight produces demonstrable, though temporary, injury to the kidneys of dogs, one might draw some rather startling, yet fairly plausible inferences as to what might be the effect of similar or much smaller doses, if the dog did not possess the ability to destroy the uric acid. From such conjectures, one might, however, also be led into gross

exaggerations. The next two experiments, Tables XVI and XVII, were made on a pure bred Dalmatian dog.

Smoky, the subject of the experiment recorded in Table XVI, was a fine Dalmatian dog, loaned us by Sergeant Lindsey Hale. The animal weighed 25 kilos, and was given 2.5 gm. of uric acid, the same quantity as we had given to most of our dogs. He became very sick after the uric acid administration. He vomited repeatedly and for several days refused all food.

TABLE XVI.

Showing Slow Destruction of Uric Acid in Dalmatian Dog—Also Temporary Kidney Injury.

	Per 100 cc. plasma.	
	Non-protein N.	Uric acid.
	mg.	mg.
1. Injected 100 mg. of uric acid per kilo in 8 min.		
2. Blood taken 10 min. before injection.	19.5	Trace.
3. " " 3 " after "	24.0	15.6
4. " " 23 " " "	21.6	8.3
5. " " 83 " " "	21.3	5.3
6. " " 3½ hrs. " "	27.0	4.3
7. " " 6 " " "	29.4	3.7
8. " " 22 " " "	41.2	1.8
9. " " 4 days " "	45.9	1.1
10. " " 8 " " "	32.7	Trace.
11. Urine of 1st day; albumin, casts, blood cells.		
12. " " 2nd " no albumin, many casts, no blood cells.		
13. Urine of 3rd day; no albumin, very few casts.		

The uric acid figures obtained from this dog are instructive. The blood of this kind of dog is not materially different from that of other dogs in that it contains under ordinary conditions only traces of uric acid. While there undoubtedly was much destruction of uric acid during and immediately after the injection, later the destructive process was entirely different from that of other dogs. In the latter, the uric acid substantially disappears in about 2 hours. In Smoky, there were (per 100 cc. of plasma) 4.3 mg. at the end of 3½ hours, 3.7 mg. at the end of 6 hours, and 1.8 mg., 22 hours after the injection.

The destruction of uric acid by Dalmatian dogs is, therefore, certainly of a different order from that found in ordinary dogs.

From the non-protein nitrogen figures for the blood plasma as well as from the albumin and casts in the urine, it is clear enough that we had obtained some form of distinct kidney injury. The non-protein nitrogen was 19.5 mg. before the injection, 41.2 mg., 22 hours later, and at the end of 4 days was 45.9 mg. The figure 32.7 mg., found at the end of 8 days, indicates recovery.

3 months later we again used Smoky for an injection experiment, but as we this time wished to determine the uric acid

TABLE XVII

Showing 80 Per Cent Recovery of Injected Uric Acid from Dalmatian Dog.

1. Daily uric acid excretion: 520, 540, 554, 510, 525, 560 mg.	Average 535 mg.
2. Daily creatinine excretion: Maximum 650 mg., Minimum 586 mg.	" 616 "
3. Total N excretion last 2 days, 7.8, 7.9 gm.	" 7.85 "
4. Uric acid excretion per (forenoon) hr. before injection.	30 mg.
5. Injected 500 mg. uric acid in 4 min.	
6. Uric acid excretion first 3½ hrs.	330 mg.—46 per cent recovered.
7. " " " next 5½ "	338 " —29 " " "
8. " " " in 24 "	933 " —80 " " "
9. " " " 2nd day.	559 " 3rd day 525 mg.
10. " " per 100 cc. plasma before injection.	0.8 mg.
11. " " " 100 " " 4 min. after injection.	6.0 "
12. " " " 100 " " 2 hrs. " "	2.6 "
13. " " " 100 " " 4½ " " "	0.8 "
14. " " " 100 " " 8 " " "	0.6 "

excretion we gave him a much smaller dose so as not to make him sick and particularly so as to avoid the complication of "nephritic" retentions of uric acid. During the intervening 3 months, as well as during the experiment, the dog was kept on a purine-free diet (milk and bread).

Some data obtained in this experiment are omitted from the table in order to facilitate inspection.

The non-protein nitrogen figures for the five blood plasmas were: 31.2, 34.5, 31.2, 32.7, and 27.9 mg., respectively. The volumes of urine per hour were 27, 218, and 135 cc., respectively; and these gave uric acid excretions per hour of 30, 99, and 52 mg. The night urine, 52 cc. per hour,

contained uric acid equivalent to only 21 mg. per hour, as against the 30 mg. per hour before the injection. The total volume of urine on the uric acid day was 2,275 cc. as against 845 and 705 cc. on the 2nd and 3rd days.

It will be noted that the uric acid recovered with the urine is given as 46 per cent for the first $3\frac{1}{2}$ hours after the injection, and 29 per cent for the urine of the following $5\frac{1}{2}$ hours, making a total recovery of 75 per cent, while the 24 hour recovery is given as 80 per cent. These figures imply that some extra uric acid was recovered with the night urine, yet the night urine contained only 21 mg. per hour. This discrepancy is due to the fact that the hourly excretion of uric acid is not uniform. The 80 per cent recovery of injected uric acid was in fact obtained during the first 9 hours. This excessively rapid elimination of injected uric acid harmonizes with the fact that this dog carried normally little, if any, more uric acid in the plasma than other dogs, yet excreted three to four times as much endogenous uric acid per kilo of body weight as does man. For further comments on this topic see pages 407 and 443.

S. R. Benedict, who discovered the unique character of the uric acid metabolism in Dalmatian dogs, found that they also eliminate much allantoin. Benedict (Harvey lecture, 1916) expresses doubt as to whether allantoin originates only as a decomposition product of uric acid. In Smoky, there was undoubtedly destruction of uric acid, yet we agree with Benedict that the origin of allantoin should not be regarded as settled. In the Dalmatian dog, the origin of uric acid also might properly be considered an open question. From the standpoint of tissue metabolism, the finding of several times as much "endogenous" purine derivatives as in man, in animals whose creatinine excretion is of the same order as that of man, points to some important missing link in the current, accepted concepts. Either the magnitude of purine metabolism in man is hidden by very extensive destructions of purine materials, or the purine derivatives, including allantoin, found in the urine of other mammals have a double origin, as in birds.

IV.

Is the rate of uric acid destruction in other animals of substantially the same order as the rate found in dogs? Are the specialized uric acid-absorbing powers of the kidney tissue and the impermeability of the other tissues a general characteristic of all animals? It seems necessary to make some experiments bearing on these questions.

The uric acid phase of protein metabolism must have undergone some remarkable evolutionary changes during the transition from the synthesis of uric acid still found in reptiles and birds, to the varying degrees of uric acid destruction found in different mammals. The power to destroy uric acid can be conceived as a process of comparatively recent origin, since it is always incomplete and since recessions such as in the Dalmatian hound can be found.⁶⁵ The impermeability of the general tissues for uric acid and the special uric acid-absorbing power of the kidney tissue, on the contrary, should be very old. This unique arrangement would clearly serve to promote the excretion of the synthesized uric acid, and therefore probably developed in response to the need for the removal of the large quantities of waste nitrogen represented by uric acid. The power to destroy the inescapable endogenous uric acid would naturally develop at a later stage and on the basis of conditions created during the period of uric acid synthesis—namely the general impermeability of the tissues and the special absorbing power of the kidneys—and therefore might be localized either in the kidneys or where we seem to find it, in the blood. The very incompleteness of the destruction indicates that it does not occur where the uric acid is produced—within the cells of all tissues. The high speed of uric acid destruction which we have found in the dog could scarcely fail to destroy every trace of endogenous uric acid, if the destroying agency were available where the uric acid is formed. Yet many dogs do excrete uric acid.

The natural normal distribution of uric acid in birds is of more than ordinary interest in connection with this discussion.

In our experiments with ducks we have determined the uric acid in whole blood instead of in the plasma. In these animals,

⁶⁵ Onslow. H., *Biochem. J.*, 1923, xvii, 334, 564.

the uric acid is usually higher in whole blood than in the plasma. The blood of ducks contains 6 to 8 mg. of uric acid per 100 cc., or 75 to 100 per cent more than normal human blood. With such high constant levels of circulating uric acid there is every opportunity for it to diffuse into the general tissues, if such diffusion could normally occur. Our accompanying figures for duck muscles (Table XVIII) show that these contain from less than 1 to 2.5 mg. of uric acid. These figures are substantially identical with the corresponding values obtained from the muscles of normal dogs, whose blood is nearly free from uric acid. In birds we have, therefore, readily accessible material on the basis of which any one can easily satisfy himself

TABLE XVIII.

Indicating: Synthesis of Uric Acid in the Liver of Birds, No Diffusion of Uric Acid into Muscle, and Great Accumulation of Uric Acid in the Kidneys.

Uric acid per 100 cc. (or 100 gm.).				
Duck No.	Whole blood.	Muscle.	Kidney.	Liver.
	mg.	mg.	mg.	mg.
1	7.3	<1	100	22.2
6	6.6	2	60	22.
7	6.8	2.5	57	18.4
8	6.2	1.7	63.5	26.2

that uric acid is not subject to free distribution between blood and tissues. The uric acid in the muscles, according to our interpretation, is only the uric acid actually produced there and bears no relation to the uric acid content of the circulating blood. The uric acid produced in muscles must, of course, get out, but it gets out not by diffusion, but by excretion—down hill excretion into the blood in the case of most mammals, but up hill excretion in the case of birds, and probably also in the case of man.

The uric acid values for our duck kidneys, 57 to 100 mg. per 100 gm., are nearly as large as the average accumulations found in the kidneys of dogs immediately after uric acid injections of 100 mg. per kilo. In the ducks there has been no sudden influx of uric acid or excessive concentration in blood. The uric acid accumulations in the kidneys prove conclusively that these accumulations are due to a specialized active power on the part

of kidney tissues to abstract and retain uric acid. To save space we have omitted many injection experiments with dogs, but it may be stated here that we have obtained even higher accumulations in the kidneys of dogs (160 mg.) by means of continuous injections, at rates so slow that the uric acid concentration in the blood never rose above 14 mg. per 100 cc. of plasma. The seemingly useless power to abstract uric acid discovered in the kidneys of dogs is revealed in ducks as a normal, active, useful function. The significance of the same uric acid-absorbing power in the kidneys of dogs is, therefore, clear enough. It is a dormant power which has not deteriorated because of long disuse and therefore is probably a fundamental, characteristic property of kidney tissue.

The high uric acid figures obtained for the uric acid in the duck livers, 18.4 to 26.2 mg., are also distinctly illuminating. These values are several times as high as the corresponding figures obtained from the livers of dogs, just as they should be, in the light of the fact, established by Minkowski, that the uric acid synthesis in birds occurs mainly or exclusively in the liver. The figures support our conclusion that the uric acid found by analysis of any fresh tissue (except the kidney) is chiefly determined by the speed of uric acid production in that tissue and is practically independent of the concentration in the blood.

The experiments so far recorded certainly show that it is quite impossible to apply to uric acid the concept of free and prompt distribution within the animal body, and this absence of distribution cannot very well be explained except on the basis of impermeability on the part of living tissues. The resistance of living tissue to uric acid can be overcome to a certain extent by means of high and sustained uric acid pressure. We have found some evidence showing a limited breakdown of the resistance to uric acid in dogs by means of continuous uric acid injections—with the help of the Woodyatt pump. And we have again encountered this phenomenon in our experiments with ducks. In a uric acid-producing animal like the duck there is no reason to expect any destruction of uric acid and we have not made any injection experiments. It did seem worth while, however, to try to see what happens when the excretion of uric acid is prevented. The kidneys of birds are so situated that it is practically

impossible to remove them without altogether too serious mutilation of the animal. But the ureters can be exposed, readily enough, and ligatured, through their opening into the cloaca without otherwise opening the animal. Birds stand either better than dogs and the operation is a slight one. Within a few minutes after such an operation the birds are seemingly as lively as before. But the operated birds do not live very long, usually less than 24 hours. It seems to us probable that they are killed by the rapidly accumulating uric acid.

The following data obtained from Duck 3 will serve to show why we suggest that the operated animals die from the effects of the accumulating uric acid.

The operation was performed at 9.30 to 10.30 in the evening. At 9.15 a preliminary sample of blood was taken from the wing veins. It contained 8.8 mg. of uric acid per 100 cc. At 11 a.m. the following morning (interval 13 hours) the animal was bled through the jugular vein and killed. This blood contained 224 mg. of uric acid per 100 cc.! (Old statements and calculations concerning the solubility of uric acid and urates in blood do not seem to apply to circulating blood.)

The other uric acid values were

	mg.
Kidney.....	354
Muscle.....	30.2
Liver.....	101

We have made a number of experiments of different kinds with ducks (and have obtained up to 400 mg. of uric acid per 100 cc. of blood) but we confine ourselves here to the experiment recorded in Table XIX. The figures there given furnish an adequate picture of the rate of uric acid accumulation in the blood of birds after ligature of the ureters. The kidneys in this case, as well as in other similar experiments, did not take up much additional uric acid. In experiments of such short duration, the kidney continues to excrete uric acid into the pelvis and attached stump of the ureter. The picture obtained is to this extent different from what would be obtained from complete removal of the kidneys.

and of the goat 92, according to Hunter. The uric acid-destroying power of the herbivorous goat should, therefore, be very nearly as great as that of the carnivorous dog.

Our first injection experiment with a goat, Table XX, showed at once that the uric acid-destroying power of this animal is entirely different from that of the ordinary dog. $1\frac{1}{2}$ hours after the injection, the blood plasma contained 9.5 mg. of uric acid; had we dealt with a dog the figure would have been about 2 mg. And at the end of 4 hours the plasma contained 1.3 mg., and this notwithstanding the fact that much uric acid was excreted. One

TABLE XX.

Showing that the Destruction of Injected Uric Acid in the Goat Is Nearly Like That of the Dalmatian Hound.

Goat 1. Weight 13 kilos.

		Uric acid.
		mg.
1. Administered 300 cc. of water by stomach tube.		
2. 1st urine by catheter (28 cc.)		Trace.
3. " blood—plasma		<0.1
4. Injected 100 mg. uric acid per kilo (1,300 mg.)		
5. 2nd blood (4 min. after injection) per 100 cc. plasma		45.6
6. 3rd " (20 " " ") " 100 " "		21.0
7. 4th " ($1\frac{1}{2}$ hrs. " ") " 100 " "		9.5
8. 5th " (4 " " ") " 100 " "		1.3
9. 2nd urine (1 hr. " ") " 68 "		370
10. 3rd " ($3\frac{1}{2}$ hrs. " ") " 150 "		352
11. 4th " (6 " " ") " 142 "		58
Total uric acid excretion in 6 hrs., 60 per cent.		780

never can tell beforehand whether a given animal will excrete any urine after the injection of as much as 100 mg. of uric acid per kilo of body weight. It all depends on how much the kidney is injured. In the case of dogs the excretion is immaterial because of the rapid destruction.

In our introductory remarks at the beginning of this section we referred to the impermeability of the general tissues for uric acid and the accompanying uric acid-absorbing power of the kidneys as an efficient arrangement for promoting the excretion of uric acid—an arrangement which, therefore, probably originated when uric acid was the chief nitrogenous waste product. One

would scarcely expect to find support for this interpretation by a study of the goat. A remarkable illustration of the efficiency of the arrangement is, however, given in Table XXI. No less than 50 per cent of the injected uric acid was eliminated in 1 hour! We doubt whether even urea could find such rapid elimination, yet, of all the waste products uric acid is considered the most difficult to excrete.

Because of the rapid excretion encountered in this experiment, the destruction of uric acid was necessarily small. The total *endogenous* uric acid excretion of the animal amounted to about

TABLE XXI.

Showing 50 Per Cent Elimination of Injected Uric Acid in 1 Hour.

Goat 2. Weight 23 kilos.

	Uric acid.
	mg.
1. Catheterized and given 300 cc. of water.	.
2. 1st blood.....	<0.4
3. Injected 2,300 mg. uric acid in 3 min.	
4. 2nd blood (5 min. after injection) per 100 cc. plasma.....	43.4
5. 3rd " (30 " " ") " 100 " "	16.8
6. 4th " (1½ hrs. " ") " 100 " "	4.6
7. 5th " (2½ " " ") " 100 " "	2.0
8. 6th " (3½ " " ") " 100 " "	1.1
9. 2nd urine (1 hr. " ") 160 cc.....	1,140
10. 3rd " (2½ hrs. " ") 55 "	332
11. 4th " (5½ " " ") 55 "	167
12. 5th " (7½ " " ") 27 "	41
13. 6th " (22½ " " ") 430 "	135
Total uric acid recovered 73 per cent.	

140 mg. per 24 hours, and the total recovery of injected uric acid was about 73 per cent. (The urines were all collected by catheterization.)

In order to obtain a somewhat more dependable basis for comparison, on the one hand between the goat and the Dalmatian dog, and on the other, between these and our human subjects we injected only 20 mg. of uric acid per kilo of body weight into another goat. Of the 272 mg. injected, 207 mg., 76 per cent, were excreted in 1 hour and 55 minutes. During the next 3 hours, another 30 mg., 11 per cent, were obtained, giving a total re-

covery of 87 per cent. From the Dalmatian dog we obtained a total recovery of 80 per cent.

The next experiment, recorded in Table XXII, may be taken to illustrate the rate of uric acid destruction in the goat, because in this case the injected uric acid caused nearly complete cessation of urine excretion. From the different uric acid figures for the blood plasma it is perfectly clear that the maximum uric acid-destroying power of the goat is of an entirely different order from that of the dog. We cannot assume that the diminishing uric acid concentrations give a perfectly correct picture of the rate of destruction. The sustained high concentrations may

TABLE XXII.

Showing True Rate of Uric Acid Destruction in the Goat. Also Temporary Nephritic Retention (Approximate Anuria).

Goat 3. Weight 10.2 kilos. Uric acid injected 1,020 mg.

Time between injection and taking of blood.	Uric acid per 100 cc. plasma.	Non-protein N per 100 cc. plasma.	Remarks.
	mg.	mg.	
1. Before injection.....	0.2	24.3	Only 4 cc. of urine obtained by preliminary catheterization, and only about 35 cc. were obtained during the first 22 hrs. after the injection.
2. 5 min. after injection.....	48.4	38.4	
3. 30 " " ".....	24.4	37.8	
4. 1½ hrs. " ".....	15.0	41.7	
5. 3¼ " " ".....	11.8	42.6	
6. 4½ " " ".....	8.2	45.3	
7. 7¼ " " ".....	5.0	55.2	
8. 24 " " ".....	1.9	99.8	
9. 48 " " ".....	1.3	146	

have forced the general tissues to take up something. Also, the preliminary filling and subsequent emptying of the kidneys might modify to a considerable extent the concentration of the circulating uric acid. In dogs, the kidneys take up their maximum during the injection, but the maximum obtainable from the dog kidney need not be its real maximum—because of the excessively rapid destruction in the blood. In the goat the situation is different. In one experiment where we injected 100 mg. of uric acid per kilo the first kidney was removed 2 minutes after the injection; it contained 138 mg. of uric acid per 100 gm., or about the same as corresponding values obtainable from dogs.

But the second kidney, removed 7 minutes after the first, contained 274 mg., twice as much, and far more than we have ever found in dogs. The second kidney was still taking up uric acid when removed, because the arterial blood passing into it just before the excision contained more uric acid than the venous blood, taken at the same time. The fall in the circulating uric acid from 48.4 to 24.4 mg. during the 25 minutes which elapsed between the second and third blood analyses in Experiment XXII does not signify, therefore, that substantially one-half of the uric acid had been destroyed during the interval. The fact that the uric acid was destroyed relatively very rapidly at the beginning and then more and more slowly is, however, quite clear. In the goat, as in the dog, the speed of the uric acid destruction is primarily determined by the circulating concentration. It will be observed that the uric acid concentration in the plasma fell only from 1.9 to 1.3 mg. during the second 24 hour period. This means only that at such concentrations of circulating uric acid the goat is just able to destroy uric acid as fast as it is produced through the breakdown of purine material. But under normal conditions goats carry only about one-tenth as high a concentration of circulating uric acid. Normally the goat, therefore, probably destroys very little uric acid.

"Uricase may be regarded as a liver ferment" (Jones⁶⁶) but for the preparation of uricolytic extracts the ox kidney seems to be used. If by any chance the goat kidney should be as efficient as the ox kidney is believed to be, then the removal of this organ should materially affect the rate of uric acid destruction. For this reason both kidneys were removed from Goat 5 before we injected the uric acid. Further, by this procedure is eliminated the one place of excessive uric acid accumulation, and we were curious to see whether the effect would not be to raise materially the level of circulating uric acid produced by the injection of 100 mg. per kilo of body weight. The results of this experiment are given in Table XXIII. The kidneys were removed under aseptic conditions, because we intended to follow the rate of non-protein nitrogen accumulation. The animal recovered well from the ether and the operation.

⁶⁶ Jones, W., *Nucleic acids; their chemical properties and physiological conduct*, London, 2nd edition, 1920.

TABLE XXIII.

Showing Rate of Uric Acid Destruction and of Nitrogen Accumulation in the Goat after Double Nephrectomy.

Goat 5. Weight 23 kilos. Uric acid injected 2,300 mg.

Time between uric acid injection and taking of blood.	Uric acid per 100 cc. plasma.	Non-protein N per 100 cc. plasma.	Remarks.
	mg.	mg.	
1. Before injection.....	0.5	58	The uric acid was injected about 2½ hrs. after the removal of both kidneys. The high original non-protein N (58 mg.) is due to unfinished recovery from a previous uric acid injection.
2. 5 min. after injection....	83.2	87	
3. 30 " " "	52.0	84	
4. 1½ hrs. " " "	37.6	75.5	
5. 3¼ " " "	25.4	77.5	
6. 7¼ " " "	13.6	95.2	
7. 20 " " "	3.2	120	
8. 24 " " "	2.3	129	
9. 2 days " " "	1.1	168	
10. 3 " " " "	1.1	246	
11. 4 " " " "	1.2	264	
12. 5 " " " "	1.3	306	
13. 7 " " " "	1.2	366	
14. 8 " " " "	1.2	420	
15. 11 " " " "	1.4	534	
16. 12 " " " "	1.3	552	
17. 13 " " " "	1.8	618	
Died at the end of 13½ days.			

About 3 hours after the operation a preliminary blood sample was taken and the urate injected (through the vein of the left hind leg). Subsequent samples of blood were then taken at exactly the same periods as in the preceding experiment so as to get strictly comparable values both for the distribution and for the rate of destruction. Some corresponding uric acid values for Goats 3 and 5 are as follows:

	5 min.	30 min.	1½ hrs.	3¼ hrs.	4½ hrs.	7½ hrs.	24 hrs.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Goat 3.	48.4	24.4	15.0	11.8	8.2	5.0	1.9
" 5.	83.2	52.0	37.6	25.4	20.4	13.6	2.3

The significance of the kidneys as a special reservoir for uric acid stands out clear and unmistakable. It is to be noted that the kidneys of Goat 3 must have held fast to absorbed uric acid for many hours—indeed even at the end of 24 hours those kidneys must still have had some uric acid left. Yet at any one period the destruction of uric acid as measured by the rate of diminution in the blood is greater in Goat 5. These data do not exclude the possibility that some uric acid destruction occurred within the kidneys of Goat 3. To secure definite information on this point it would be necessary to repeat the experiment until another goat responded to the uric acid injection with approximately complete anuria and then determine the uric acid content of the kidneys $7\frac{1}{2}$ or 24 hours after the injection.

In one experiment, in which nearly complete anuria occurred, we have obtained some additional data proving that the kidneys of goats actually do retain uric acid for a long time. The goat used in this experiment weighed 26 kilos, and was given 2,000 mg. of uric acid intravenously. The first kidney was removed 15 minutes after the end of a 4 minute injection. It contained 154 mg. of uric acid per 100 gm. At the end of 5 hours the last sample of blood was taken and the second kidney was removed. The plasma contained 2.5 mg. of uric acid while from the kidney we obtained no less than 228 mg. per 100 gm. It is clear that the second kidney of this goat held fast to the absorbed uric acid far more tenaciously than do the kidneys of dogs and it seems reasonable to assume that the difference is due to the incomparably greater speed of uric acid destruction in the blood of the dog.

In the light of these results it would, of course, be interesting to know whether the uricolysis experiments applied to the kidneys of oxen would give negative results with the kidneys of goats. But we have become more and more skeptical as to the validity of conclusions concerning metabolism processes based on reactions demonstrated by means of tissue extracts. We would recall in this connection the rapid transformation of the urea of excised muscles into ammonium salts.⁶⁷

Goat 5 lived almost exactly 2 weeks after the double nephrectomy. The animal ate liberally of hay and oats for about 1 week and

⁶⁷ Gad Andresen, K. L., *Biochem. Z.*, 1921, cxvi, 266.

much carbonate accumulated in the blood. The equilibrium between the production and the destruction of uric acid became stationary at about 1.2 mg. per 100 cc. of plasma, but rose to 1.8 mg. the day before the animal died. The creatinine content of the plasma rose in 4 days to 29 mg., and in 6 days to 42 mg., and then became stationary at the latter figure. At this level we have seemingly encountered another equilibrium—between the production and the destruction (or alteration) of creatinine.

A few data obtained from a cat and some rabbits are worth recording to show that the distribution of uric acid is probably of the same order in all animals. A cat and a rabbit were given intravenously 100 mg. of uric acid per kilo of body weight. 4 minutes after the injection blood was taken and 2 minutes later the animals were killed and the tissues taken for analysis. The uric acid values obtained were as follows:

	Blood.	Muscle.	Kidney.	Liver.
	mg.	mg.	mg.	mg.
Cat.....	18	4.6	62	14
Rabbit.....	38	3.7	66	12

These figures show that the distribution of injected uric acid is substantially similar in the cat and rabbit as in the dog and the goat. They also indicate that the speed of uric acid destruction is of about the same order in the cat as in the dog while the destruction in the rabbit is very much slower—probably of about the same order as in the goat.

The fact that the cat and the dog show very rapid destruction of uric acid while the rabbit and goat reveal a very slow process indicates, of course, that rapid destruction is a characteristic of carnivorous animals.

As our supply of goats was rather limited we have used rabbits for the study of one or two additional questions of considerable importance. The main question may be stated as follows: The uric acid-absorbing power of kidney tissue represents a normal, active function in birds. Is the same power completely dormant in other animals? Is the normal excretion of endogenous uric acid by herbivorous animals accomplished on the basis of the uric acid concentrations in their blood plasmas without any

preliminary concentration of uric acid in the kidney tissue? This is manifestly an important question in connection with attempts to explain just how kidneys accomplish excretion. The current view that excretion occurs on the basis of the circulating waste products without intermediary concentration in absorbing kidney cells may be entirely erroneous. Folin and Berglund have suggested that the excretion of sugars is probably determined by the concentration in the kidney tissue rather than by the concentration in the blood. This line of reasoning should be especially applicable to the common waste products, and particularly to uric acid, since we now know that kidney cells really possess a specialized power of absorbing this waste product.

Uric acid determinations in normal rabbits should show whether uric acid excretion is accomplished by filtration plus subsequent water absorption or whether a preliminary concentration in the kidneys analogous to that of birds may be involved.

From two normal rabbits the following uric acid values were obtained.

	Plasma.	Muscle.	Kidney.
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
Rabbit 2.	1.1	1.4	3.4
" 3.	0.9	1.2	2.8

We do not take the position that these uric acid figures definitely prove that the rabbit kidneys have absorbed and concentrated circulating acid just as the duck kidneys were doing, but the values obtained certainly are in harmony with such an interpretation. Rabbit plasma contains distinctly more uric acid than the plasma of the goat. According to our point of view these higher levels in the rabbit signify that the rabbit excretes uric acid less efficiently than the goat and the difference manifestly can be due to a somewhat inferior responsiveness on the part of the uric acid-absorbing power of rabbit kidneys. This phase of the uric acid problem could not have been anticipated until we had begun to realize that herbivorous animals probably destroy very little endogenous uric acid and by that time most of the work reported in this paper had been completed. Before

making a comprehensive study of the relative concentrations of uric acid in normal kidneys it probably will be necessary to determine whether the uric acid color reaction made directly on the kidney extracts is sufficiently specific for this more exacting purpose.

In order to prove that the kidneys of herbivorous animals as a matter of fact do function as a place of storage and concentration for uric acid under conditions which are not far removed from the normal, we have injected very small quantities of uric acid. Into Rabbits 4 and 5, weighing about 2.5 kilos, we injected 5 and 10 mg., respectively, and then waited 1 hour before killing the animals. The uric acid values obtained were as follows:

	Plasma.	Muscle.	Kidney.
	mg.	mg.	mg.
Rabbit 4.....	1.6	1.4	4.2
" 5.....	2.4	1.6	6.9

It is to be noted that in each of these rabbits, as also in the preceding two strictly normal ones, the uric acid concentration in the kidneys is just about three times as great as the concentration in the blood plasma. While we recognize that the uric acid content of normal kidneys stands in need of more investigation there is no doubt in our minds as to the significance of the uric acid values found in the kidneys of these four rabbits.

Because the circulating uric acid levels in our rabbits were several times as high as those of our goats it seemed worth while to sacrifice one normal goat in order to get a picture of the uric acid distribution between blood, kidneys, and muscles of this animal. The analytical results obtained are as follows:

	mg.
Plasma.....	0.4
Muscle.....	1.0
Kidney.....	3.4

In connection with the absorbing power of kidneys it remains to be stated that all the kidney analyses reported in this paper

were made on the cortex. For uric acid we have also looked into the question of its distribution between the cortex and the medulla and in the dog we have found even more uric acid in the pyramids than in the cortex. Our first attempts to make such regional analyses were not very satisfactory, because the removal of the capsule and the opening of a highly edematous kidney involves unavoidable loss of much fluid. By first ligating the pedicle and then freezing solid ($-15^{\circ}\text{C}.$) the whole kidney, with its capsule, perfect separation of medulla and cortex without any loss of liquid was accomplished.

The results obtained from such analyses have not been of exactly the same order in the goat as in the dog. The analyses of the goat's kidneys referred to on page 411 included separate analyses of cortex and medulla. The first kidney contained a concentration of 154 mg. of uric acid in the cortex and 166 mg. in the medulla. The second kidney, taken 5 hours later, gave a uric acid concentration in the cortex of 228 mg., and the same value, 232 mg., for the medulla. On the other hand, from a dog kidney, removed 2 minutes after a rapid injection, the frozen cortex gave 124 mg., per 100 gm., and the frozen medulla, 312 mg.

The specific uric acid-absorbing power of kidneys revealed in this paper suggests, of course, the possibility that other waste products may be subjected to a similar preliminary concentration process. Important as the question is in relation to interpretations of the kidney function we confine ourselves here to one brief statement, based on half a dozen different experiments: The kidneys absorb both urea and creatinine from the circulating blood, but the resulting concentration within the cortex of the kidneys is relatively small—rarely amounting to more than twice that in the corresponding blood plasma.

V.

Many of the experiments reported in this section were made before we had done any work on animals. It was only because the results obtained from our human subjects clearly indicated a very imperfect distribution of the injected uric acid to the tissues that we finally decided to inquire whether the distribution ascertainable in dogs could supply the needed information. How

much of the information gathered from our experiments with animals will prove directly applicable to the uric acid problem as it pertains to man is perhaps not for us to say, or for any one to say, at the present time, but the discussion here presented reflects, necessarily, a point of view which could not have been formulated on the basis of experiments with human subjects alone.

Intravenous injections in man of from 0.5 to 2 gm. of uric acid in the form of almost neutral lithium urate solutions represent a comparatively simple and safe procedure. The uric acid was purified by several recrystallizations, and was sterilized by dry heat (110°C., 2 hours) before being dissolved by means of separately sterilized water and lithium carbonate solution. 0.4 gm. of lithium carbonate is adequate for the solution of 1 gm. of uric acid at 60°C. These solutions were always made immediately before the injection. The lithium carbonate under these conditions destroys no uric acid, and, at least in the quantities used, has none of the toxic properties of piperazine. Since uric acid solutions of the required degree of concentration, 1 to 2 per cent, can be prepared by the help of lithium carbonate, it is unfortunate that piperazine, with its confusing superimposed effects, was ever introduced in connection with the administration of uric acid.

In most of our experiments we have injected approximately 22 mg. per kilo of body weight (10 mg. per pound). This is not a very large amount. If it were actively taken up by the tissues, as are the amino acids, the residuum left in the blood should sink to a few tenths of a milligram above the initial level. On the other hand, if the distribution were like that of urea, we might fairly expect to be left with an increase in the blood plasma of not less than 2, nor more than 4, mg. at the end of the necessary short distribution period. If either of these two possibilities had been found to correspond to the experimental facts we doubtless should have increased the uric acid dosage in later experiments; but, as it turned out, the amounts given are more than ample to secure unmistakable analytical figures, both for blood and for urine, and larger doses would only increase the danger of injury to the subjects.

A preliminary experiment made with only 0.5 gm. of uric acid

had, in fact, shown that we would encounter no difficulties in the way of finding accumulations of uric acid in the blood. In this case, the second sample of blood was drawn from one arm while the urate solution was still running into the veins of the other, but this precaution was entirely unnecessary. The subject, H.B-d, was on a mixed, but purine-free and low nitrogen, diet throughout the 3 weeks duration of the experiment, and in the course of this period received two 2 gm. injections of uric acid, in addition to the preliminary 0.5 gm. injection. The last injection was accompanied by the administration of atophan. The main experiment in which 2 gm. of uric acid were injected is recorded in Table XXIV.

The figures there shown reveal most vividly the unexpected results obtainable from intravenous injections of uric acid in man. In this case the uric acid of the blood plasma rose from 5.6 mg., immediately before the injection, to 22.4 mg., immediately after—an increase of 16.8 mg. It may be remarked that such levels for the uric acid content of blood very nearly equal the highest figures obtained for the latest stages of nephritis. During the following 10 minute period, allowed for the establishment of equilibrium between blood and tissues, the level in the plasma fell only 2.2 mg.—to 20.2 mg. This subject weighing 88 kilos should have had about 4 liters of plasma, and this plasma contained 224 mg. of uric acid before the injection, 896 mg. at the end of the injection, and 808 mg. at the end of another 10 minutes. In other words, during the first 10 minutes, counting from the beginning of the injection, 1,328 mg. of the administered 2,000 mg. of uric acid had been removed from the blood, while during the second 10 minute period only 88 mg. were removed—and this, notwithstanding the fact that the level in the plasma at the end of that period still stood at the extraordinary figure of 20.2 mg. per 100 cc.

These figures clearly show that the available tissue reservoir for uric acid in man is extremely limited. The absorption of 1.3 gm. of uric acid by the tissues of this man weighing 88 kilos clearly resulted in a strain on their absorptive capacity. The distribution of 1.3 gm. to 50 kilos of tissue material would mean an average absorption of only 2.6 mg. per 100 gm. of tissue. Variations greater than that are found in the plasma of normal

TABLE XXIV.
Showing Prolonged Retention in Blood and 47 Per Cent Loss (Destruction) of Injected Uric Acid.
 Subject H. B.-d. Age 36 years; weight 88 kilos.

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc. mg.	Non- protein N per 100 cc. mg.	Volume.		Uric acid.		
			Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.	
Feb., 1923.			cc.	cc.	mg.	mg.	Since Jan. 23 on a purine-free low protein diet.
2				730		608	Average endogenous uric acid 605 mg.
3				568		568	
4				648		630	
5							
11.12 a.m.	5.6	20.1	67		21		2.0 gm. uric acid intravenously.
11.13-21 a.m.							
11.23 a.m.	22.4	24.8					
11.33 "	20.2	22.6	99		286		
11.51 "							
12.12 p.m.	13.1	22.3					
12.50 "			73		120		
1.47 "			55		82		
2.17 "							
4.10 "			48		72		
5.51 "			56		73		
12.00 m.			67		60		
8.00 a.m.			26		38		Uric acid recovered: 748 mg. 37.4 per cent. 212 " 10.6 " " 107 " 5.3 " " Total...53 "
6	8.7	21.9		1,230		1,353	
7	6.3	21.9		478		817	
8				568		712	
9				562		630	
				540		630	

men under ordinary conditions. To us it seems quite unreasonable to assume that the major human tissues should normally be so nearly saturated with uric acid that they cannot take up a few extra milligrams per 100 gm. We conclude rather that the taking up of uric acid, or the holding of uric acid, is not normally a function of the major tissues of man. To draw any other conclusion would involve the assumption that the distribution of uric acid in man is fundamentally different from the distribution found in animals. The conclusion that muscles do not take up uric acid does not imply that all the uric acid which has disappeared from the blood has found lodgement in the kidneys. We have seen that the resistance of the muscles to uric acid is not so absolute and complete but that they can be forced to take up some uric acid in response to the sustained pressure of very high concentrations in the blood. We assume that in this respect human tissues do not differ materially from the tissues of other animals.

Our hypothesis that injected uric acid cannot escape into the bulky general tissues (muscles) is not based only on the high levels remaining in the plasma after a relatively short distribution period. The extraordinary length of time during which those levels remain very high is even more significant. During the first 38 minutes after the injection the subject of Table XXIV eliminated uric acid at the rate of 286 mg. per hour, yet at the end of 1 hour the uric acid in the plasma stood at 13.1 mg., and at the end of 3 hours it stood at 11.8 mg. The fall of 1.3 mg. during the intervening 2 hour period can manifestly be due to the excretion. At the end of 27 hours the uric acid concentration was 8.7 mg., a level which we might reasonably have expected to find within 10 minutes after the injection. Even at the end of another day the uric acid was unmistakably high, 6.3 mg., instead of the initial value of 5.6 mg.

From the uric acid figures cited, it is clear that a single moderate intravenous injection of uric acid in man will produce uric acid levels in the blood which are unexpectedly high as well as unexpectedly persistent. How do these higher levels affect the uric acid excretion? How much is ultimately recovered in the urine? How much, if any, is destroyed?

The subject had been on a purine-free diet containing only 30 gm. of protein for over a week before the uric acid injection, and during that period the 24 hour, as well as many hourly, uric acid eliminations had been determined. In response to the injected uric acid the hourly uric acid output rose promptly from 21 to 286 mg., while the uric acid in the plasma rose from 5.6 mg. to a maximum of at least 22.4 mg., and again sank—probably to something like 16 mg. This high speed of elimination lasted only for a few minutes. The second urine, representing 50 minutes, revealed a rapid fall in the excretion, the average for this period being 120 mg. per hour. The third urine (57 minute period) represents nearly the same uric acid concentration in the plasma as the preceding period, yet the hourly excretion is only 82 mg.—a fall of 38 mg. During the night (12 to 8 a.m.) the average excretion was 38 mg. per hour. The total extra uric acid excreted during the first 21 hours amounted to 37.4 per cent of that injected. The 2nd day (24 hours) yielded 10.6 per cent more, and the 3rd, 5 per cent—53 per cent in all.

It is possible that the next two 24 hour urines also contained traces of "extra" uric acid, but the figures obtained do not warrant any such assumption, and the point is immaterial, for, at all events, it could be only a matter of a few milligrams. It is clear that in this experiment only about one-half of the injected uric acid was recovered. The other half disappeared. So far as we can see it is not possible to interpret this disappearance except on the basis of destruction.

The figures recorded in Table XXV are, in all essential respects, substantially identical with those discussed in connection with Table XXIV. The subject, C.D-k, weighed less—67 kilos, and the amount of uric acid injected was, therefore, only 1,450 mg., instead of 2 gm. The following points may be noted. The uric acid levels in the blood at the end of 10 minutes, as well as immediately after the injection, were within 2 mg. of the corresponding figures obtained from H.B-d. It is rather fortunate that these two subjects, joint authors of this paper, gave so nearly identical values, notwithstanding their very different weights and physical characteristics, because this fact removes the necessity of considering whether we might not be dealing with subjects who are not strictly normal. Whether the subjects are strictly

normal or not could not materially alter the significance of the results obtained, but the figures for the non-protein nitrogen of the blood show certainly that we were not dealing with subjects having abnormal nitrogen retentions. The point of normality is one which we mention here only because these two subjects, as a matter of fact, represent the highest degree of immediate uric acid accumulation which we have found.

That the excessive height of uric acid level reached in these two subjects was not accidental is proved by the fact that each subject later received a second injection of the same quantity of uric acid (atophan experiments) as was used the first time, and in each case, the second experiment gave exactly the same early levels as had been obtained from the first injection. One remarkable feature to be noted in Table XXV is that the uric acid level in the plasma was the same on the morning of the 2nd day after the injection as at the end of the first 21 hours (8 mg.). From this table one can also see, rather more clearly than from the first, that there is no close correspondence between the uric acid level in the plasma and the uric acid excretion. The hourly excretion just before the injection was 26 mg., the 1st night urine after the injection yielded only 21 mg. per hour, notwithstanding the continuing high level in the plasma.

Another point to which attention might be called is the fall in the non-protein nitrogen following the uric acid injection. It fell steadily from 19.5 mg., the initial value, to 15.6 mg., about 6 hours later. This depletion of the circulating non-protein nitrogen is presumably due to the excessive diuresis. Varying degrees of diuresis are always obtained from moderate urate injections, but only in some is it associated with a fall in the non-protein nitrogen. The diuresis is seemingly without effect on the uric acid excretion; but in nearly all cases the water excretion is so abundant that the concentration of uric acid is less than 2 mg. per cc. of urine. Special experiments, involving copious water drinking, have confirmed our finding that "water diuresis" does not produce a more rapid excretion of the injected uric acid. The diuresis following uric acid injection most probably should be regarded as a symptom of a transient injury to the kidneys.

The fall in the non-protein nitrogen of this subject is par-

ticularly interesting from one point of view. In man, whose destruction of uric acid is of an entirely different order from that of dogs, the injection of 20 mg. per kilo of body weight might produce just as extensive accumulations in the kidneys as does the injection of five times as much in the case of dogs. And accompanying such an accumulation there should then occur the same swelling and the same temporary injury—in short, a temporary but, nevertheless, distinct “nephritis.” The injection of 20 mg. of uric acid per kilo does not seem to produce any nephritic symptoms in normal human subjects. Most of the subjects become somewhat sick for a few hours, some very much more than others, but in no normal person have we obtained any definite signs, except the diuresis, of even a most temporary kidney injury. Whether the human kidney is especially resistant to the effects of an influx of uric acid we do not know, but there is no reason to doubt that a given injection of uric acid per kilo of body weight should produce greater and more persistent accumulations in the kidneys in the case of man than in the case of the dog. The accumulation should be more like that of the goat. Too much stress probably should not be laid on our failure to find signs of a temporary injury. Here, as in the case of dogs, the surplus capacity of the kidneys is large, and considerable damage to the organs would have to be done before one could expect to find at least the symptoms of nitrogen retention, especially since the subjects were on a low protein diet.

The uric acid excretion recorded in Table XXV is of the same order as in the first experiment. The total amount recovered in Experiment XXV cannot be calculated very exactly, because of uncertainty as to how much to allow for endogenous uric acid. The subject had been on the same purine-free diet as H. B-d, and for the same length of time. The average daily uric acid output was 461 mg. before the injection, yet during the 6 days following the injection the output fell consistently until it was only 366 mg. The extra output of injected uric acid might perhaps be safely figured at 55 to 60 per cent; 36 per cent for the 1st day, 10 per cent for the 2nd, and about 10 per cent for the following 2 days. The significant fact to be noted is that, on the 4th day after injection, there was still some of the added uric acid circulating in the blood and contributing to the excretion.

In order to set forth the extraordinary variations occurring in different normal individuals with respect to injected uric acid, the reader's attention is next called to the figures recorded in Tables XXVI and XXVII. The experimental conditions were the same as for the first two subjects, except that a little more carbohydrate food was taken. In all four cases, the diet was purine-free and very low in protein. These two subjects gave substantially identical figures for the uric acid in the plasma at corresponding periods. In both cases we failed to find the initial very high concentrations recorded in Tables XXIV and XXV, but if we compare the levels existing at the end of about half an hour, all four are very much alike.

Table XXIV = 13.1 mg., Table XXV = 12.7 mg., Table XXVI = 11.6 mg., Table XXVII = 11.2 mg.

Or if we subtract the uric acid present before the injection, so as to get the increases due to the added uric acid, we have the following.

Table XXIV, 7.5 mg.; Table XXV, 7.3 mg.; Table XXVI, 6.6 mg.; Table XXVII, 6.8 mg.

The plasma values found the following day are also very similar, the figures being 8.7, 8, 7.8, and 7.2 mg., respectively. The differences encountered in the elimination, and therefore also in the disappearance (destruction) of uric acid, are, however, very great. The excretions for the first 2 days in each case are as follows:

Table XXIV	=	37.4	+	10.6	=	48	per cent.
" XXV	=	36	+	10	=	46	" "
" XXVI	=	59	+	13.5	=	72.5	" "
" XXVII	=	19	+	9	=	28	" "

As already stated a little additional uric acid doubtless does come out after the 2nd day, but the amount is small and difficult to calculate with exactness.

The subject, H. B-n, of Table XXVII is probably the most vigorous and healthy subject of all, in so far as any such comparison could be made. But he became quite sick, and thoroughly incapacitated for several days after the injection. He was familiar with the work and knew that some of our subjects did not stand

TABLE XXVI.
Showing Sustained Excretion and Only 9 Per Cent Destruction of Uric Acid.
 Subject H-w-d. Age 26 years; weight 62 kilos.

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		
			Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.	
May, 1923.	mg.	mg.	cc.	Per 24 hrs.	mg.	mg.	Total N per 24 hrs.
1						gm.	
2				1,280	552	4.2	
3				1,320	508	4.1	
4				1,000	462	3.9	
5				1,210	494	3.9	
11.40 a.m.	5.0	19.5	90		25		
11.42-46 a.m.							
11.57 a.m.	12.8	21.9	219		335		
12.29 p.m.	11.6	22.2	286		145		
1.20 "			264		67		
2.00 "	9.8	21					
3.35 "			84		58		
5.03 "	9.5	19.2	66		74		
10.30 "			83		93		
8.00 a.m.			36		25		
				1,845	1,302		
6	7.8	18.9		1,000	686	3.9	Uric acid recovered: 798 mg. 59 per cent.
7	6.1	20.1		620	600	3.8	182 " 13.5 " "
8	5.1	23.4		880	650	4.4	96 " 7 " "
9	5.3	21		1,030	546	4.0	146 " 11 " "
10				1,325	560	4.2	Total...91 " "
11				1,300	491	3.9	

TABLE XXVII.
Showing About 7% Per Cent Destruction of Uric Acid.
 Subject H. B-n. Age 29 years; weight 70 kilos.

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		
			Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.	
July, 1923.	mg.	mg.	cc.	cc.	mg.	mg.	Total N per 24 hrs.
7							gm.
8							5.3
9							6.1
11.29 a.m.	4.4	21.6	24		18		Average endogenous uric acid 489 mg.
11.31-34 a.m.							
12.03 p.m.	11.2	23.1	148		84		1,500 mg. uric acid intravenously.
12.48 "			327		43		
1.43 "							
2.20 "	10.4	24.9	200		43		
2.34 "			118		33		
4.40 "							
4.50 "	9.3	22.5	128		40		
10.00 "			91		24		
7.30 a.m.							
10	7.2	24.9		2,557		774	5.1
11	5.6	23.1		1,760		625	5.5
12	5.2	21.9		1,300		519	5.4
13	4.6	26.7		1,020		462	5.1
				1,420		480	5.7

the injection so very well. He therefore preferred to get first a small injection, and he had received 400 mg. 4 days before the one recorded in Table XXVII. He suffered no ill effects at all at that time, but the larger subsequent dose proved very severe in result. He lost several pounds in weight, in the course of the following few days.

The recovery of uric acid recorded in Table XXVI is our best on a low protein diet. In this case there was unmistakably a considerable extra uric acid elimination during the 3rd and 4th days as well, so that the total recovery was fully 90 per cent. If all individuals reacted in approximately the same way as this subject, H-w-d, first year medical student, one might feel justified in accepting the prevalent view that uric acid is substantially indestructible in the human organism. It is to be noted, however, that the speed of elimination in H-w-d was extremely rapid, 59 per cent during the first 21 hours as against only 19 per cent in Table XXVII.

From this marked difference in the speed of elimination, particularly during the 1st day, one might conclude that it is the rate of excretion which determines the degree of destruction. The more rapid and extensive the elimination, the less would be left for destruction. In favor of this conclusion one might point to the fact that the kidneys of different normal individuals seem to exhibit different degrees of efficiency since the blood, or plasma, of different normal persons contains such widely different levels of uric acid under ordinary conditions. While the speed of elimination must affect the degree of the destruction, the speed of destruction must also affect the total excretion—yet both these processes, as we shall see, are subject to independent variations.

The following points may be noted in connection with these two experiments. Subject 26 not only eliminated three times as much extra uric acid the 1st day as did Subject 27, but the former continued to excrete extra uric acid during 3 additional days, while the latter stopped at the end of 1 additional day. Had there been no destruction of uric acid or had there been equal speed of destruction, B-n, Table XXVII, should have shown the longer period of excretion. The results obtained indicate therefore that B-n *destroyed* uric acid much more rapidly than H-w-d,

and so far as concerns these two subjects, there is no occasion for also ascribing diminished power of elimination to B-n, since the slow and short elimination may have been a consequence of the rapid destruction. The uric acid figures obtained for the blood of the two subjects are in harmony with the view that the predominant and decisive differences have to do with their respective destructive capacities for uric acid. Before the injection, B-n's plasma contained 4.4 mg., H-w-d's, 5 mg. This difference is not great nor very significant, but the following morning B-n had 7.2 mg. in the plasma, and H-w-d, 7.8 mg. These figures clearly suggest that B-n had somehow got rid of just as much uric acid during the intervening period as did H-w-d; and as B-n excreted a small amount, he presumably destroyed a great deal.

There is another point which might well be referred to in connection with Tables XXVI and XXVII. We have obtained abundant evidence in the course of our numerous experiments that uric acid produces more or less severe, though seemingly temporary, toxic symptoms. Mr. B-n, as previously stated, was nearly prostrated a few hours after receiving the injection and it took him a long time to recover. H-w-d, on the other hand, remained perfectly well, had probably less discomfort than any one else, and did not object to being used again. This great difference in the toxic effects is decidedly suggestive in view of the fact that one subject got rid of the uric acid mainly by excretion. Since the former became ill, it looks as if the toxic symptoms were due to the decomposition products of uric acid rather than to uric acid itself. It is not possible, of course, to measure or compare the subjective symptoms. These symptoms (aside from the immediate minor and fleeting feelings of congestion in the head) come 3 to 4 hours after the injection, and consist of headache, nausea, and an increasing feeling of weakness bordering on exhaustion. Also the subject looks decidedly pale. Much more work must be done before one can say with any degree of certainty whether the toxic effects obtained from uric acid injections are due to uric acid itself or to its decomposition products. And our main problem here is necessarily to elucidate only the fact that decomposition of uric acid *does* occur within the human organism.

We believe that the results recorded in Tables XXIV to XXVII prove conclusively that it is no longer possible to deny that there is destruction of uric acid in man, but more experiments bearing on a point concerning which there has been so much controversy are appropriate.

The results shown in Table XXVIII and XXIX are different from any of those found in Tables XXIV to XXVII. From the blood analyses we learn that there is an unusually rapid removal of the uric acid from the circulation. In 40 minutes the level has sunk from an unknown maximum to 10 and 10.9 mg., respectively. In another couple of hours we have the levels at 8.6 and 8.4 mg.; and the following morning the uric acid has reached the low levels of 5.7 and 5.5 mg. The corresponding figures in the first four tables range between 7.2 and 8.7 mg. Notwithstanding this unusually rapid disappearance of uric acid from the blood during the first 24 hour period it is clear, at least from Table XXVIII, that the small residuum still in the blood is removed very slowly. On the 3rd day, the subject of this table, W-n, another medical student, eliminated 115 mg., or 8 per cent of the injected uric acid.

B-ck, Table XXIX, on the other hand, probably eliminated no uric acid on the 3rd day. Since 62 per cent was eliminated by B-ck the 1st day, against 48 per cent by W-n, and since the total elimination of these two subjects is identical, 65 per cent, it seems reasonable to assume that in the case of B-ck, we have encountered greater speed, both of destruction and of elimination, than in W-n. B-ck's speed of elimination is greater even than that of H-w-d, Table XXVI, 62 *versus* 59 per cent, during the 1st day, yet the total excretion of B-ck is only about two-thirds that of H-w-d. It clearly is not possible to interpret such differences except on the basis of different speeds, both for the destruction and for the elimination of injected uric acid—after one has once accepted destruction as a feature of the intermediary metabolism of uric acid.

The figures recorded in Tables XXIV to XXIX probably give a correct but, of course, incomplete picture of what happens to uric acid injected into the human circulation. The variations encountered are surprisingly large considering the uniformity of the experimental conditions, but these variations

TABLE XXVIII.
Showing Loss (Destruction) of About 35 Per Cent of Injected Uric Acid.
Subject W-n. Age 25 years; weight 62 kilos.

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		
			Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.	
Apr., 1923.	mg.	mg.	cc.	cc.	mg.	gm.	Since Apr. 2 on a purino-free low protein diet.
9				820		4.1	Average endogenous uric acid 425 mg.
10				670		3.9	
11				370		3.1	
12				655		4.1	
13							
11.46 a.m.	4.2	21.6	47		35		1,350 mg. uric acid intravenously.
11.47-52 a.m.							
12.02 p.m.	12.7	20.7	400		264		
12.29 "							
12.32 "	10.0	21	191		107		
2.00 "	8.6	20.4	400		77		All afternoon the subject had thirst, headache, chills--no discomfort next day.
2.09 "					84		
2.48 "			672				
3.38 "							
5.00 "	7.5	19.2	99		41		
10.00 "			14		19		Uric acid recovered: 651 mg. 48 per cent. 125 " 9 " " 115 " 8 " " Total.. 65 " "
8.00 a.m.				2,400		4.8	
14	5.7	18.3		1,400	1,076	3.4	
15	5.1	19.8		1,000	550	4.9	
16	4.7	19.5		1,050	540	4.2	
17				835	485	3.9	
18	4.3	19.8			465		

1,350 mg. uric acid intravenously.

All afternoon the subject had
thirst, headache, chills—no dis-
comfort next day.

TABLE XXIX.

Indicating Both Rapid Elimination and Rapid Destruction of Uric Acid.

Subject B-ck. Age 25 years; weight 77 kilos.

Time.	Plasma.		Urine.				Total N per 24 hrs.	Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.			
			Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.		
	mg.	mg.	cc.	cc.	mg.	mg.	gm.	
May, 1923.								
16								Average endogenous uric acid 570 mg.
17				2,610		539	5.4	
18				2,300		524	5.3	
19				3,900		617	6.1	
20				3,240		599	5.0	
9.58 a.m.	4.3	18.6	183		24			1,600 mg. uric acid intravenously.
10.00-04 a.m.								
10.14 a.m.	12.5	20.1	39		145			
10.38 "	10.9	18.9	115		150			
12.06 p.m.	8.4	18.3	180		138			
12.14 "								
3.28 "	8.2	18.6	47		55			Uric acid recovered: 987 mg. 62 per cent. 55 " 3.5 " " 48 " (?) Total...65.5 per cent.
3.38 "			91		38			
10.45 "								
8.00 a.m.								
21	5.5	17.4		2,151		1,557	5.2	
22				1,310		635	5.0	
23				2,300		618	5.1	
24	4.5	20.1		2,320		540	4.9	
25				2,820		579	5.1	
26				2,060		541	4.3	
27	4.5	20.1		1,720		520	3.9	Received 800 mg. lithium chloride intravenously. This produced no effects of any kind.
				2,210		540	4.9	

do not hide the two significant facts that uric acid lingers for a very long time in the blood stream, and that large amounts of uric acid can be destroyed within the human organism.

The experiments recorded in Tables XXX to XXXV confirm and extend the observations. The normal uric acid elimination in man is subject to large and seemingly unaccountable variations, no matter how uniform the diet or what kind of a diet is used. A most interesting characteristic of the normal uric acid elimination is that it is so easily modified by conditions other than the feeding of its precursors (purines).

Tables XXX and XXXI illustrate the effect of urea together with a low nitrogen diet on the excretion of injected uric acid. (The results obtained from the same subjects, H-w-d and B-ck, on a low protein diet alone are shown in Tables XXVI and XXIX.) The urea (50 gm.) was given by mouth, 15 gm. before the uric acid injection, and the remainder in divided doses at different intervals.

H-w-d had eliminated 59 and 13 per cent, respectively, on the 1st and 2nd days, a total of 72 per cent. Under the added influence of 50 gm. of urea the corresponding values were 73 and 18 per cent or a total of 91 per cent. During the 3rd and 4th days, without urea, he eliminated 7 and 11 per cent, respectively, while in the urea experiment the corresponding figures are 5.5 and 4.5 per cent. The final total of 101 per cent in the urea experiment can scarcely be accepted as showing complete recovery of the uric acid injected. It is more probable that we have happened to encounter more than the average amount of endogenous uric acid excretion during those 2 days. If one were to accept the view that the recovery represents only injected uric acid one would have to conclude also that in this case no destruction of uric acid took place. While H-w-d's destruction undoubtedly was small both in Experiment 26 and in Experiment 30, Table XXXIV shows that H-w-d destroyed up to 26 per cent of injected uric acid when on a high protein diet, and it is therefore scarcely reasonable to assume that he destroyed no uric acid at all in the experiment under discussion.

The second subject, B-ck, Table XXXI, had shown even higher speed of elimination than H-w-d on the low protein diet alone, but in this experiment the urea failed completely to produce an

TABLE XXXI.
Showing that Urea Has Little or No Effect on Uric Acid Excretion.
 Subject B-ok. (See Tables XXIX and XXXIII.)

Time.	Plasma.		Urine.					Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		Total N per 24 hrs.	
			Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.		
	mg.	mg.	cc.	cc.	mg.	mg.	gm.	
23-27								See Table XXIX. Average endogenous uric acid 570 mg. (?). Took 15 gm. urea. 1,600 mg. uric acid intravenously. Took 15 gm. urea; on May 30 took 20 gm. urea. Uric acid recovered: 797 mg. 50 per cent. 216 " 13 " " 56 " (?) Total....63 per cent.
29								
10.06 a.m.	4.2	17.1						
10.08 "								
10.50 "	4.2	25.2	307		30			
10.54-58 a.m.								
11.32 a.m.	10.1	31.5	226		290			
11.51 "								
1.04 p.m.	8.6	27.6						
1.15 "								
1.29 "			208		122			
3.13 "			248		89			
4.04 "								
5.45 "	7.5	35.1						
8.00 a.m.			288		60			
			146		37			
				4,680		1,367		
				3,940		786		
				4,000		626		
				1,690		465		
30								
1	5.6	23.1						
2	4.2	16.2						

acceleration or total increase in the uric acid elimination. Lewis and Doisy, using smaller doses, found that urea has no influence on the hourly output of endogenous uric acid.

In all of the eight experiments recorded above the subjects had been kept on a very low protein diet. This plan was adopted because it was thought that the endogenous uric acid output would be not only at a minimum, but also more constant, under such conditions, and we hoped, therefore, that we should be able to determine with greater certainty the exact amount and per cent of recovered uric acid. The uniformly low nitrogen excretions and the low levels of the non-protein nitrogen in the plasma give a clear picture of the underlying dietetic condition. From this diet we hoped to secure not only a minimum of endogenous uric acid excretion, but also the lowest attainable levels of circulating uric acid in the plasma. The different uric acid values obtained before the injections show that the last named object was not attained. The values vary between 4.2 and 6.0 gm. and give an average of 4.9 mg. per 100 cc. of plasma. The average non-protein nitrogen was only 19.8 mg.

These figures are decidedly significant. In the following three tables (XXXII to XXXIV) we have the corresponding values for the uric acid and non-protein nitrogen levels on a purine-free, but protein-rich diet. The average non-protein nitrogen level is here 28.3 mg., instead of 19.8, while the uric acid level is only 3.4 mg. as against the 4.9 mg. obtained on the low protein diet. Notwithstanding the marked increases in the endogenous uric acid excretion obtained from the purine-free, but protein-rich diet, there is an unmistakable fall in the level of the circulating uric acid.

These unexpected findings lead to one or two important conclusions. They show that there is no definite relationship between the level of the circulating uric acid and the magnitude of the uric acid excretion even under practically normal conditions—just as we have failed to find any close relationship between the uric acid level in the plasma and the speed of the elimination after the injection of uric acid.

The fact that a high protein diet produces a larger excretion of endogenous uric acid and at the same time reduces the circulating level of uric acid below the levels obtainable on low pro-

tein diets would seem to furnish a new point of view for the dietetic treatment of persons with a gouty disposition. It has long been recognized that such persons should abstain from food which is rich in purines and it has also been thought advisable to reduce the endogenous production of uric acid by a sparing use of every kind of protein material. But our findings seem to indicate that the best dietetic method for reducing the circulating uric acid should be a purine-free diet containing enough protein to yield 15 to 20 gm. of nitrogen in the urine. The dietetic literature bearing on gout has probably greatly exaggerated the importance of abstaining from all food which contains any purine materials. A small amount of purine products should be of very little consequence provided that the diet is distinctly high in protein.

Our suggestion concerning dietetic treatment in gout probably has very little bearing on those severe cases where the subjects, because of the frequency of the attacks, are more or less completely incapacitated for work. It refers particularly to those more common subjects who have had few attacks and whose kidneys are nearly normal in their capacity to eliminate urea.

The low uric acid levels in the blood and simultaneous high uric acid output obtained on purine-free, high protein diets would seem to suggest that the most prominent effect of such diets is to promote the elimination of the endogenous uric acid. By virtue of the accelerated elimination resulting in a corresponding diminution in the destruction, the extra output of endogenous uric acid obtained under these conditions could be adequately explained, without assuming that there is any increase in the production of endogenous uric acid on high protein diets. The results obtained from uric acid injections are, unfortunately, not consistent enough to prove definitely that this interpretation is correct. Excessive speed of elimination is indeed shown by B-ck, in Table XXXII—76 per cent of the injected uric acid came out in the course of 21 hours, as against 62 per cent during the corresponding period on a low nitrogen diet (Table XXIX), and 50 per cent on the low nitrogen diet plus urea (Table XXXI). W-n, Table XXXIII, eliminated 60 per cent of the uric acid on the high protein diet as against 48 per cent on the low protein diet—in 21 hours. From H-w-d, on the other hand, we got 57

TABLE XXXII.
High Protein Diet Tends to Promote Excretion of Uric Acid.
 Subject B-ck. (See Tables XXIX and XXXI.)

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		
			Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.	
Mar., 1923.	mg.	mg.	cc.	cc.	mg.	mg.	Total N per 24 hrs.
13				2,550		599	18.7
14				2,530		624	19.0
15				2,060		584	16.7
16				2,580		579	18.1
17				2,360		625	17.9
18							
10.39 a.m.	3.0	30.9	18.5		33		Average endogenous uric acid 602 mg.
10.40-47 a.m.							
10.58 a.m.	9.5	28.2					
11.26 "	6.6	28.5					
12.10 p.m.			125		343		
1.10 "	5.7	29.7	217		134		
2.06 "							
5.05 "	3.6	28.8	147		105		
5.10 "			157		85		
10.00 "			88		25		
8.00 a.m.				3,190			
19	4.6	25.5		2,630		1,824	Uric acid recovered: 1,222 mg. 76 per cent.
20				4,000		787	185 " 11 " "
						625	Total...87 " "

Uric acid recovered:
 1,222 mg. 76 per cent.
 185 " 11 " "
 Total...87 " "

1,600 mg. uric acid intravenously.

TABLE XX.XIII.
High Protein Diet Tends to Promote Excretion of Uric Acid.
 Subject W-n. (See Table XXVIII.)

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		
	mg.	mg.	Per hr.	cc.	Per hr.	Per 24 hrs.	
Feb., 1923.						Total N per 24 hrs.	Since Feb. 14 on a purine-free high protein diet.
19						gm.	
20				2,530		524	
21				1,240		535	
22				1,780		530	
23				1,815		490	
24				1,650		385	
11.47 a.m.	3.8	27.3	46		29		Average endogenous uric acid 493 mg.
11.52-56 a.m.							
12.05 p.m.	12.0	31.2					
12.51 "	7.9	29.4	145		222		
2.08 "	6.9	27.0					1,350 mg. uric acid intravenously.
2.15 "			205		96		
4.10 "			164		75		
4.40 "	6.3	28.5					
11.00 "			91		65		Uric acid recovered: 808 mg. 60 per cent. 85 " 6 " " 85 " 6 " " Total...72 " "
8.00 a.m.			102		30		
	5.1	26.4		2,475		1,303	
25	3.4	28.5		1,105		580	
26				1,400		580	
27				1,840		540	
28				1,800		500	

TABLE XXXIV.
High Protein Diet Tends to Promote Excretion of Uric Acid.
 Subject H-w-d. (See Tables XXVI and XXXV.)

Time.	Plasma.		Urine.				Remarks.	
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.			
			Per hr.	cc.	Per hr.	Total N per 24 hrs.		
Mar., 1923.	mg.	mg.	cc.	cc.	mg.	gm.		
11						570	15.6	
12						580	15.6	
13						645	17.0	
14						655	17.6	
15								
10.03 a.m.	3.4	26.7	33		31		Average endogenous uric acid 613 mg.	
10.12-16 a.m.								
10.25 a.m.	11.1	29.4						
10.54 "	9.6	31.8						
11.00 "								
12.00 m.			142		244		1,350 mg. uric acid intravenously.	
12.26 p.m.			340		146			
2.02 "	7.6	29.4	130		52			
4.10 "	6.5	32.7	164		74			
10.00 "			149		54			
8.00 a.m.			60		33			
16	5.7	30.3	2,627		1,372	16.9	Uric acid recovered:	
17	4.4	28.2	1,330		840	17.0	764 mg. 57 per cent.	
18			1,380		655	16.1	232 " 17 " "	
19	4.1	28.5	1,400		570	14.9	47 " (?)	
			1,580		640	16.4	Total... 74 per cent.	

TABLE XXXV.
High Protein Diet Tends to Promote Excretion of Uric Acid.
 Subject H-w-d. (See Tables XXVI, XXX, and XXXIV.)

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		
	mg.	mg.	Per hr.	cc.	Per hr.	mg.	
Mar., 1923.							Since Mar. 20 on a purine-rich high protein diet.
24							Average daily uric acid 1,304 mg.
25				2,400		1,292	
26				2,360		1,275	
27				3,280		1,250	
28				1,980		1,400	1,350 mg. uric acid intravenously.
10.48 a.m.	5.4	29.4	45		70		
10.55-11.05 a.m.							
11.10 a.m.	14.4	32.4			400		
11.38 "			175				
11.40 "	11.3	31.8			154		
12.50 p.m.			188				
1.07 "	9.5	32.1			184		
2.19 "			179		112		
3.57 "			159				
4.00 "	7.9	33.6					
10.00 "			150		90		Uric acid recovered:
8.00 a.m.			55		56		921 mg. 68 per cent.
29	6.2	28.2		2,455		2,225	246 " 18 "
30	5.8	27.1		1,400		1,550	
31	5.2	28.8		1,660		1,330	Total...86 per cent.
				2,300		1,330	

per cent recovery on the high protein diet, or practically the same figure as the 59 per cent given by the low protein diet.

The figures, therefore, seem to indicate that, in H-w-d, the high protein diet failed to produce an increased elimination. From the *total recovery* of uric acid with the two diets we are, however, compelled to conclude that H-w-d had little destruction of uric acid while on low protein diet and that he, for some reason, was destroying uric acid much faster 2 months earlier when on the high protein diet. There is every reason to believe that the peak of the destruction as well as the peak of elimination of uric acid occurs in the earlier periods after the injection of uric acid. When thus considered from the standpoint of simultaneous destruction and excretion of uric acid, it seems justifiable to conclude that the elimination figures obtained from H-w-d with the high protein diet do not contradict our inference that high protein diets tend to increase the speed with which uric acid is eliminated. The point involved here is of considerable theoretical importance aside from its practical bearing on the dietetic treatment of gouty persons. The increased endogenous uric acid excretion on high protein diets first found by Folin, in 1905, and since repeatedly verified by others, has never been explained.

To us it seems quite reasonable and probable that high protein diets accelerate the speed of uric acid elimination—it has long been known that high protein diets produce increased elimination of administered creatine. Because of this increased speed of excretion the circulating uric acid is kept at a lower level and because of this lower level there is less destruction. Here we have a seemingly adequate and simple explanation of why more endogenous uric acid is eliminated on high protein diets. The conspicuous fluctuations in the excretion of endogenous uric acid both on high and on low protein diets are due to fluctuations in the two variables—elimination and destruction. The temporary increases in the uric acid excretion obtained from the injection of simple amino acids, by Lewis and Doisy, represent most probably an increased elimination and nothing more. To explain the increased output on the basis of increased production would imply a responsiveness on the part of the kidneys to minute increases in the circulating uric acid which the kidneys do not

possess. Sodium chloride in large doses, 30 gm., can accelerate the uric acid excretion quite as much as amino acids.

The figures given in Table XXXII are particularly interesting. The uric acid in the plasma stood at 3 mg. before the injection, and in the course of about 6 hours fell to 3.6 mg. or almost the initial value. In none of our numerous injection experiments made on man while on low protein diets have we seen anything approaching such a rapid disappearance of the uric acid from the blood. The results obtained in this experiment resemble those obtained with the Dalmatian dog, Table XV. In the first $3\frac{1}{2}$ hours, B-ck excreted about 36 per cent; Smoky, the dog, about 46 per cent. In about $9\frac{1}{2}$ hours, Smoky eliminated 80 per cent, while B-ck, in $10\frac{1}{2}$ hours eliminated over 60 per cent. The blood figures also are of the same order in these two different subjects. 11 minutes after the injection, the circulating uric acid in B-ck was 6.5 mg. higher than before the injection, while in Smoky, the increase 4 minutes after the injection was 5.2 mg. And about 2 hours after the injection the extra circulating uric acid was 2.7 mg. in B-ck, and 1.8 mg. in Smoky. The speed of destruction as well as the speed of elimination has been unmistakably more rapid in Smoky than in B-ck, but the difference is of about the same order as the difference between B-ck and W-n in Tables XXXII and XXXIII.

It should be noted in connection with Table XXXII that B-ck had a higher level of circulating uric acid, 4.6 mg., 21 hours after the injection than he had at the end of 6 hours, 3.6 mg. This would seem to indicate that the release of the uric acid stored up in the kidney comes much later in man than in dogs, as should be the case, since the total removal by excretion plus destruction is so much slower in man than in the dog.

The results shown in Table XXXV are the outcome of our endeavor to increase by heavy feeding with normal purine-rich food the store of uric acid in the body before injecting the uric acid. This preliminary object, as we see, was not attained, at least so far as can be seen from the uric acid level in the plasma. The *exogenous* daily uric acid output rose from nothing to about 700 mg., but the plasma level rose only to 5.4 mg. The accompanying high protein content of the food has been, almost certainly, a factor in promoting the elimination of the heavy

load of exogenous uric acid. The effects of the superimposed uric acid injection are nearly identical with those obtained from the same subject on the high nitrogen, but purine-free diet, the only difference being that about 10 per cent more uric acid was recovered on the high purine diet.

The twelve experiments with normal subjects recorded in this section show conclusively that destruction of uric acid does occur within the human organism. This fact probably would have been established long ago if the problem had not become obscured by a premature acceptance of the theory that destruction of uric acid within the animal organism is confined to its transformation into allantoin. It is easy enough to understand how that theory came to escape adequate critical study. It explained at once both the origin of allantoin and the occurrence or absence of uric acid, and thus seemed to fit all the known facts. It is certainly a remarkable coincidence that human urine should be the only mammalian urine (except perhaps that of the ape) which contains no allantoin and that human blood should contain from ten to twenty times as much uric acid as the blood of other mammals (except the sea lion, *Swain*).

The allantoin problem does not come within the scope of our investigation; but we must attempt to formulate some explanation of the truly characteristic high uric acid contents of human blood.

It is certain that the high levels of circulating uric acid in man could not occur if the oxidation processes for uric acid were as highly developed in man as they are, for example, in the goat. A relatively slow oxidation must be accepted as an established fact. The difference in this respect between man and the goat though not very large, is certainly very much smaller than the difference between the goat and the dog; but that difference must be large enough to permit the continuance in human blood of the uric acid concentrations actually found there.

A far more important difference between man and the other mammals we believe to be represented in their different capacities to excrete uric acid. It is solely because of man's inferior power of excretion that human blood contains almost as much uric acid as the blood of birds. The power of the human kidney to excrete uric acid is of an inferior order and is also subject to

great variations. Our most efficient normal subject (B-ck, Table XXXII) fell unmistakably behind the one Dalmatian dog available for investigation and fell far behind the goat (page 407). Quite aside from conclusions based on injection experiments we might refer to the enormous excretion of "endogenous" uric acid by the Dalmatian dog—this excretion was three times as great in proportion to body weight as is obtainable from normal men, yet the uric acid in the plasma remained at the ordinary low levels of 0.1 to 0.3 mg. For one reason or another the human kidney has become vastly inferior to the kidneys of the four footed animals in relation to the excretion of uric acid.

At present, it is scarcely possible to go much beyond this bare statement of facts. In relation to uric acid the kidney function may be divided into two separate processes, preliminary absorption and subsequent excretion. The inefficiency of the ~~human~~ kidney, therefore, can be a lack of sensitiveness in either process. To us it seems rather more reasonable to assume that the responsible factor is lack of sensitiveness on the part of the uric acid-absorbing power. Different degrees of sensitiveness on the part of this kidney function would explain admirably why there is no relationship normally between the concentrations of circulating uric acid and urea, or between the uric acid concentration and the speed of elimination. The responsiveness is different in different persons and that is why each individual tends to carry his own level of circulating uric acid, quite independently of whether he destroys much or little. The apparent specificity in uric acid retentions thus would seem to be best explained on the basis of the demonstrably specific uric acid-absorbing power of kidney tissue. If this absorbing process were sufficiently responsive to very low concentrations of passing uric acid no accumulations could occur, except in advanced nephritis, because the increasing accumulations in the kidneys would eventually compel excretion.

From analyses of nephritic bloods we know that the blood uric acid can rise to something like 20 or 25 mg. At these highest levels the uric acid destruction is probably just about equal to the production. The corresponding value in the goat, after double nephrectomy, becomes stationary at above 1.2 mg. per 100 cc. of plasma. In dogs, and presumably in other carnivorous

animals, there is no uric acid accumulation following double nephrectomy. In birds, as we have seen, ligating the ureters raises the circulating uric acid to from 200 to 400 mg. Birds die before their non-protein nitrogen has risen above 200 mg., man at 300 to 350 mg., while dogs and goats live until levels of about 600 mg. have been attained.

We permit ourselves here to make a few additional remarks concerning the destruction of uric acid. There is no reason to doubt that this destruction is produced by oxidation. Further, if our conclusion is correct, that in the dog this oxidation takes place in the blood, one cannot reasonably assume that the oxidation in man or in other animals takes place elsewhere than in the blood. Corresponding to, and responsible for, this oxidation, some oxidizing agent or some condition producing active oxygen must be available, just as something of the same sort must be postulated for the oxidations in tissues of ordinary food materials. The strange point is only the long discredited idea that any such oxidations may take place in the blood. The fact that this unknown oxidizing mechanism should be so much more abundant or effective in the blood of the dog than in the blood of man (or the goat) is, of course, decidedly disconcerting.

While recognizing this difficulty, we cannot get away from the fact that it is just in the dog, in which the oxidation is so rapid, that we have been compelled to ascribe it to the blood. It may well be that other instances of more effective oxidations in the dog than in man will be found. The oxidations of acetone bodies in diabetic dogs may be referred to in this connection. Also it must not be forgotten that the mechanism of oxidation within the animal body is still unknown.

If the uric acid is really oxidized in the blood one must necessarily wonder about the nature of the process and particularly whether any other oxidations occur in the same place. It is possible that the oxidation is produced through the agency of some specific enzyme though we have failed to find any evidence pointing in that direction. On the other hand, one must not entirely overlook the possibility of less specific and more or less unregulated oxidations. Many organic substances to which the animal organism has never had a chance to adapt itself are oxidized in whole or in part. To what extent such foreign materials

reach the living cells of tissues may be considered an open question. So far as we can see it is by no means excluded that the oxidations of such substances, like that of uric acid, take place in the blood. We recognize that the assumption of unregulated oxidations is open to the objection that ordinary food materials would be exposed to the same conditions during their passage through the blood. But we are not sure whether this is or is not a weighty objection. From the specific dynamic action of foods, it would appear that the influx of food is, in fact, accompanied by mysterious increases of oxidations. These extra oxidations may be considered as unregulated, at least in the sense that they depend on the intake rather than on any recognizable body need. In this respect they resemble the oxidation of uric acid, the speed of which, in any one animal, depends primarily on the circulating concentration.

VI.

In our historical review we omitted the literature on gout. It seems necessary to make here a few observations on the modern literature on this subject; especially since most of the prominent writers on gout rather recently have expressed their revised views.

Schittenhelm⁶⁸ is seemingly the only one whose views on gout still include the assumption that there is destruction of uric acid in man. He is rather vague as to the rôle of this destruction in gout. He seems to have abandoned the older view of Brugsch and Schittenhelm that there is a general retardation, in gout, of all the intermediary steps in the metabolism of the purines. The most important feature in the modern German theories of gout is that the general tissues have an especial affinity for uric acid. Injected uric acid is actively absorbed by these tissues. Gudzent⁶⁹ pictures the process as a case of real uric acid fixation to which he has given the name "Uratohistechie." This theory of Gudzent's is more or less the same as the historetention theory whereby Umber⁷⁰ tried earlier to explain the chief cause of trouble in the gouty. These views of active tissue fixation ignore the high levels of circulating uric acid in gout.

The Brugsch-Schittenhelm theory has been abandoned because of the work of Thannhauser and his coworkers. Thannhauser and Czoniczer⁷¹

⁶⁸ Schittenhelm, A., and Harpuder, K., *Z. ges. exp. Med.*, 1922, xxvii, 43.

⁶⁹ Gudzent, F., *Berl. klin. Woch.*, 1921, lviii, 1401.

⁷⁰ Umber, F., *Deutsch. med. Woch.*, 1921, xlvii, 216, 245.

⁷¹ Thannhauser, S. J., and Czoniczer, G., *Z. physiol. Chem.*, 1920, cx, 307;

have determined not only the uric acid, but have also "determined" combined purines (nucleotides) in blood serum. The "normal" nucleotide values obtained from the serum of gouty blood are interpreted as proving that the Brugsch-Schittenhelm theory of retarded purine metabolism cannot be correct. It must be admitted that this evidence against the Brugsch-Schittenhelm theory is as obscure as was the theory itself. From Jackson's⁷² careful work it would appear that the nucleotides of blood are located in the blood cells and not in the plasma—an important difference from the standpoint of interpretation. Benedict's⁷³ combined uric acid is also confined to the blood cells. Having proved to his own satisfaction that uric acid is the only purine present in abnormal amounts in the blood serum of the gouty, Thannhauser goes right back to Garrod for his interpretation of gout. "Gout is a constitutional, often inherited, inferiority of the kidney to excrete uric acid, an inferiority which does not extend to the excretion of other waste products"—a very familiar point of view.

The facts and points of view presented in the preceding sections must necessarily serve as a starting point for study of the uric acid metabolism in gout. Abnormally high levels of circulating uric acid are more frequent in this disease than are demonstrable urate deposits. But those high levels can no longer signify that the whole organism is loaded with uric acid. The general tissues of the gouty should contain very little if any more uric acid than the tissues of normal persons. On the other hand, it is impossible to get away from the fact that urate deposits do occur in many persons who have gout. These deposits occur only on cartilages, in certain bone tissues and some connective tissues—in which the circulation is usually very poor.

This remarkable distribution of urate deposits can scarcely be due to anything else than that the tissues involved differ from the general tissues in their permeability to uric acid. Granted permeability, and the diffusion of uric acid, however slow, would produce urate concentrations equal to those of the plasma. If these concentrations exceed the solubility, precipitation can take place; without saturation, precipitation is impossible. Low temperatures would lower the solubility, and deposits are never found except in localities that are rather poorly protected against cold. Whether the diffusion of urates into the tissues where deposits occur is a normal phenomenon or a consequence of an

⁷² Jackson, H., Jr., *J. Biol. Chem.*, 1923, lvii, 121.

⁷³ Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 633. Davis, A. R., Newton, E. B., and Benedict, S. R., *J. Biol. Chem.*, 1922, liv, 595.

alteration in the tissues of the gouty is an important question. To solve this question it is necessary to determine whether the uric acid content of normal and gouty joint fluids exhibit only the same quantitative differences as are found in the corresponding blood plasmas—or whether the differences are virtually qualitative, in the sense that gouty joint fluids contain much uric acid and the normal, none.

The fluids obtained from inflamed and swollen joints of the gouty probably always contain substantially the same uric acid concentrations as the blood plasma. A striking illustration of this point was obtained from one patient, Gl-y, Table XLVI. This patient was having an acute attack while under investigation. From two preliminary blood samples we had obtained uric acid values (plasma) of 8.6 and 8 mg. The day before the injection we removed the fluid from the left knee; its uric acid concentration was 8.9 mg. The next day, 5 hours after the uric acid injection, the knee was again punctured. The fluid now contained 14.8 mg. of uric acid while the plasma, taken at the same time, contained 16 mg. These joint fluids, however, contained about 5 per cent of albumin, as well as some fibrinogen; the finding of uric acid in such fluids proves nothing concerning the diffusion of urates into joints which are not subject to the infiltration of blood proteins.

This research represents in no sense an endeavor to explain the complex factors which go to produce the acute attacks of the gouty; nor have we considered the tophi, except in connection with the diagnosis. Urate deposits must be secondary to, and an ultimate consequence of, the high levels of circulating uric acid. Our study is confined, in the main, to an elucidation of the significance and immediate consequences of the high uric acid in the blood of the gouty.

Clinical Data on Gouty Subjects of Experiments XXXVI to XLVI.

Medical No. 21061, P.B.B.H., (Tables XXXVI and XXXVII).—Mr. G. Th. K-s; age 38 years; weight 81 kilos. Diagnosis: gout. For the last 10 years has had, about twice a year, acute gout attacks of 2 to 3 months duration. These involved originally only the great toe, but later became polyarthritic in distribution. Tophi in left ear. Roentgen examination: arthritic changes approaching those characteristic of gout. Urine: occasionally had very slight trace of albumin and a few hyaline casts.

Medical No. 22896, P.B.B.H., (Table XXXVIII).—Mr. J. J. B-th; age 48 years; weight 50 kilos. Diagnosis: *gout*. For 9 years has had periodic attacks of 3 to 4 weeks duration. Early attacks in left great toe; later attacks, polyarthritic, with swelling and pain in the joints. Tophi in ear. Roentgen examination: arthritic changes not characteristic. Urine: no albumin when admitted.

Medical No. 23231, P.B.B.H., (Table XXXIX).—Mr. J. Br-k; age 53 years; weight 72 kilos. Diagnosis: *gout*. For 12 years has had recurrent attacks in great toes and ankles. The last attack just previous to admission had lasted 10 weeks. This was his fourth severe attack. No tophi. Roentgen examination: slight, and not characteristic, arthritic changes. Urine: normal.

Medical No. 23038, P.B.B.H., (Table XL).—Mr. J. F. G-er; age 70 years; weight 50 kilos. Diagnosis: *gout*. For 10 years has had typical gout attacks in great toe joints of both feet. These attacks would begin in the morning and lasted only about 2 hours. Roentgen examination: multiple arthritic changes combined with punched out areas characteristic of gout. Ears studded with tophi. Urine: normal. Moderate hypertension.

Medical No. 23179, P.B.B.H., (Table XLI).—Mr. J. S-ds; age 68 years; weight 70 kilos. Diagnosis: *gout*. First attack 27 years ago—in both feet. 10 years ago other joints became involved. A few tophi in both ears. Roentgen examination: a lesion in great toe suggestive of gout. Urine: trace of albumin and some finely granular casts. Hypertension; chronic myocarditis.

Medical No. 21380, P.B.B.H., (Table XLII).—Mr. Ch. E. B-l; age 41 years; weight 98 kilos. Diagnosis: *gout*. 3 months ago had acute attack in left great toe. This attack lasted 10 days. Right great toe was tender. Tophi in both ears. Roentgen examination: slight arthritic changes—not definitely characteristic for gout.

Medical No. 22874, P.B.B.H., (Table XLIII).—Mr. F. M-th; age 52 years; weight 64 kilos. Diagnosis: *gout*. First attack 10 years before, in great toe; lasted several days. Later attacks extended to other joints. Tophi in both ears. Roentgen examination: arthritic changes of uncertain character. The fluid from left knee contained 3,680 white cells per c.mm.—mostly polynuclear.

Dr. S-n, private case, (Table XLIV).—Age 29 years; weight 87 kilos. Diagnosis: *gout*. 1½ years ago had typical podagra in right great toe. This attack began during the night; it lasted 2 weeks. Typical swelling, redness and soreness. During next 3 months had two other similar attacks. No tophi. Urine: no albumin or casts.

Medical No. 23189, P.B.B.H., (Table XLVI).—Mr. W. J. Gl-y; age 56 years; weight 97 kilos. Diagnosis: *gout*. Had first attack 3 years ago in right great toe. Somewhat later other joints became involved. Admitted 3 weeks after the beginning of an acute attack. One tophus in right ear. Roentgen examination: nothing indicative of gout. Additional diagnosis: chronic myocarditis; auricular fibrillation.

TABLE XXXVI.
Showing Over 80 Per Cent Destruction of Uric Acid in Gout.
 Subject K-z. (Compare with Table XXXVII.) Diagnosis: gout.

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		
			Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.	
May, 1923.							Since May 2 on a purine-free low protein diet.
6			ca.	ca.	mg.	mg.	Average endogenous uric acid 350 mg.
7				930		384	
8				670		312	
9				980		360	
10				930		357	
11				1,270		347	
1.15 p.m.							1,100 mg. uric acid intravenously.
1.16-19 p.m.	10.7	24.9	32		16		
1.29 p.m.	18.0	24.9					
2.10 "	14.3	23.4					
2.30 "			245		75		
4.47 "	13.7	22.2					Uric acid recovered: 166 mg. 15 per cent. Total...15 per cent.
4.50 "			37		34		
7.00 a.m.			51		17		
12	13.6	23.4		1,350		516	
13				1,410		355	
14	11.2	23.1		1,100		363	
				940		369	
							4.3
							4.0
							3.7
							4.0

TABLE XXXVII.
Showing Rapid Destruction of Uric Acid.
 Subject K-z. Age 38 years; weight 81 kilos. Diagnosis: gout.

Time.	Plasma.		Urine				Remarks.
May, 1923.	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		Total N per 24 hrs.
	mg.	mg.	Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.	
			cc.	cc.	mg.	mg.	gm.
19				1,560		353	12.1
20				1,450		353	12.3
21				1,670		410	12.1
22							
10.13 a.m.	8.0	34.8	35		23		Average endogenous uric acid 372 mg.
10.21-24 a.m.							
10.40 a.m.	14	34.2	77		93		
11.37 "	12.7	33.6			51		
11.47 "			204		42		1,100 mg. uric acid intravenously.
2.00 p.m.	11.0	60.6	195		15		
4.25 "			63				
4.31 "							
7.00 a.m.							
23	10	31.5		2,216		638	11.4
24				1,170		460	11.1
25				1,300		409	11.0
				1,320		405	11.9
							Uric acid recovered: 266 mg. 24 per cent. 88 " 8 " " Total...32 " "

dividuals. But the total elimination remains extremely poor, something like 70 per cent of the injected uric acid has disappeared and by far the greater part of this must have been destroyed. It is interesting to note that in the preliminary period the high protein diet has also increased the level of endogenous uric acid excretion, from 350 mg., on the low protein diet, to 372 mg., and has lowered the level of the circulating uric acid from 10.7 to 8 mg.

The next gouty subject, B-th, Table XXXVIII, received substantially as much uric acid per kilo as our normal men; he suffered no discomfort. Here, as in the preceding two experiments, there can be little room for doubt about the significance of the analytical results. Of the 1,200 mg. injected, 253 mg., 21 per cent, came out the 1st day, and there is no justification for assuming that the subsequent urines contained any of the administered uric acid. The circulating uric acid remained high for several days, 10.5 mg., as against 7 mg. before the injection, but the gouty kidney is even less sensitive to unmistakable changes in the levels of circulating uric acid than are the kidneys of normal persons.

The gouty person, Br-k, Table XXXIX, served as subject shortly after an attack which had lasted for several weeks. He received 1,400 mg. of uric acid, and responded with an excretion of 165 mg., 12 per cent, during the 1st day. In the table we have assigned further recoveries of 2 and 3 per cent for the 2nd and 3rd days. We have done so mostly on the basis of the fact that the uric acid in the plasma was still distinctly high, 4.5 mg. above the preliminary level, at the end of 24 hours. Unresponsive as all gouty kidneys are to changing levels of circulating uric acid, an increase of 4.5 mg. should have had some effect. It is impossible in the cases of many of the gouty to determine what value to take for the endogenous excretion, because it is much more variable than in normal individuals.

From the points of view developed in connection with our experiments with normal persons, the results obtained from the three gouty subjects of Tables XXXVI to XXXIX seem to us perfectly clear. These subjects all had a rather high speed of uric acid destruction, but probably not as high a speed as that of B-n, Table XXVII. Except for the condition of their kidneys,

TABLE XXXVIII.
Showing at Least 75 Per Cent Destruction of Injected Uric Acid.
 Subject Mr. B-th. Age 48 years; weight 50 kilos. Diagnosis: gout.

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		
			Per hr.	cc.	Per hr.	mg.	
Jan.-Feb., 1924.	mg.	mg.	Per 24 hrs.	cc.	Per 24 hrs.	mg.	Total N per 24 hrs.
24	9.2	34.2		1,180	302	10.5	Jan. 24-29 on purine-free high protein diet. From Jan. 30 on a low protein purine-free diet.
25				1,500	335	12.3	
26				1,480	307	12.7	
27				1,590	273	14.1	
28	7.8	54.0					Acute gout attack. Urine: no albumin.
4				1,040	258	5.2	Urine: very slight trace albumin. Jan. 31 and Feb. 1: 3 gm. atophan were given a day.
5							
11.42 a.m.	7.0	32.7	41		10		
11.44-46 a.m.							
12.02 p.m.	18.2	32.4	125		89		1,200 mg. uric acid intravenously.
1.20 "							Average daily uric acid 352 mg.
1.32 "	15.1	30.9	69		29		
4.10 "							
5.30 "	12.7	35.7	56		23		
9.45 "			66		20		Uric acid recovered: 21 per cent.
6.00 a.m.							Urine: no albumin.
6	11.7	31.5		1,435	605	5.6	
7				970	380	5.4	
8	10.5	30.8		820	380	5.3	
9	10.3	30.0		1,080	390	5.6	
				1,000	343	4.1	

TABLE XXXIX.
Showing Not Less Than 80 Per Cent Destruction of Injected Uric Acid.
 Subject Mr. Br-k. Age 53 years; weight 70 kilos. Diagnosis: gout.

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		
			Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.	
Mar., 1924	mg.	mg.	cc.	cc.	mg.	mg.	Total N per 24 hrs.
10							6 m.
11				1,200		355	9.0
12				870		355	8.6
10.57 a.m.	6.5	31.0	34		19		Average uric acid per day 355 mg.
11.08-11 a.m.	15.1	32.4					
11.25 a.m.	12.7	32.1					1,400 mg. uric acid injected intra- venously.
1.05 p.m.			81		48		
1.10 "			18		11		Uric acid recovered: 12 per cent. 2 " " 3 " " Total...17 " "
4.30 "	12.0	36.0	91		20		
6.00 a.m.				1,640		520	5.5
13	11.0	38.1		1,800		387	7.3
14				1,540		400	7.9
15	9.8	41.4		1,010		313	7.1
16				1,370		420	8.9
17				1,060		337	
18	5.2	25.8					

TABLE XI.
The Normal Recovery of Injected Uric Acid Here Suggests a Subnormal Process of Destruction.
 Subject Mr. G-cr. Age 70 years; weight 50 kilos. Diagnosis: gout.

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		
			Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.	
Feb., 1924.	mg.	mg.	cc.	cc.	mg.	mg.	From Feb. 14 on a "purine-free" low protein diet.
14							Average daily uric acid 244 mg.
15							
16							
11.09 a.m.		50.4	40		13		
11.11-13 a.m.	7.2			530		202	1,000 mg. uric acid intravenously.
11.28 a.m.	15.2	51.6		625		287	
12.58 p.m.	12.8	58.8					
1.30 "			62		54		
4.35 "	11.7	57.1	29		18		
6.00 a.m.			57		19		
				1,215		500	Uric acid recovered:
17	9.6	49.2		745		327	25.6 per cent.
18				600		280	8.3 " "
19	9.2	54.6		485		198	3.6 " "
20				490		210	" "
22	8.1	45.6					Total..37.5 " "

Previous and Subsequent History.—Jan. 5, 1924, patient was seen in an acute attack of gout. Plasma uric acid was 14.7 mg., and non-protein N 34.5 mg. per 100 cc. Atophan was given, but discontinued more than 1 week before admission to the hospital. Feb. 25 and 26, patient suffered some diffuse joint pains. Atophan was given and on Mar. 1, the plasma contained 6.8 mg. uric acid and 72.0 mg. non-protein N per 100 cc. On Mar. 5, the corresponding figures were 9.2 and 58.2 mg., respectively.

TABLE XLII
Showing Normal Recovery of Injected Uric Acid—Probably Subnormal Power of Destruction.
 Subject Mr. S.-ds. Age 68 years; weight 70 kilos. Diagnosis: gout.

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		
			Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.	
Mar., 1924.	mg.	mg.	cc.	cc.	mg.	mg.	Since Mar. 4 on a "purine-free" low protein diet.
6	8.9	48.3		1,350		395	Average uric acid per day 406 mg.
7				1,500		380	
8				940		423	
9				1,030		427	
10							
10.05 a.m.	8.0	33.3	32		14		1,400 mg. uric acid intravenously.
10.07-09 a.m.							
10.25 a.m.	16.1	35.4	80		71		
11.37 "	14.9	34.8	35		35		
11.58 "	12.7	42.3	50		29		
4.00 p.m.							Uric acid recovered: 20 per cent. 9.5 " " 9.3 " " 2.1 " " Total....40.9 " "
6.00 a.m.							
11	10.5	39.9		1,118		705	
12				1,010		560	
13	9.8	46.8		940		555	
14				980		455	
15	9.0	47.1		1,180		435	
16				1,180		370	
17				1,130		397	
18	6.7	39.3		1,500		367	

by virtue of which they were compelled to carry high levels of uric acid, they would undoubtedly have excreted at least as much (probably more) of the injected uric acid as did B-n. But because of those high levels, the actual destruction is greater.

For the two subjects, G-er, Table XL, and S-ds, Table XLI, we have given the recoveries of administered uric acid as 37.5 and 40.9 per cent, respectively. These figures are, in a measure, arbitrary, because here, as in the preceding cases, the endogenous excretion was so uncertain. It is clear, however, that these two subjects also illustrate our point that the gouty tend to *destroy* more, and to *excrete* less uric acid than normal persons. While these two have in fact excreted more uric acid than did B-n, Table XXVII, this is because their speed of uric acid destruction is intrinsically of a much lower order, a fact indicated by the longer period (3 days) during which extra uric acid came out.

B-l, Table XLII, was one of our first gouty subjects and received only 10 mg. per kilo. The results obtained are worth recording, because they show so clearly that in the gouty, as in normal persons, a large part of the injected uric acid lingers in the blood stream until excreted or destroyed. The uric acid level was 7.4 mg. before the injection and 9.6 mg. 24 hours later. It is impossible to say how much was excreted and how much destroyed. We give the recovery of the 1st day as 28 per cent, but on the same basis of calculation the 3rd day should correspond to another recovery of 25 per cent. This might possibly be interpreted as the so called delayed excretion in gout, but we have no faith in the concept of delayed excretion, for we do not believe that the injected uric acid has found any unusual reservoir or hiding place in this subject. The uric acid excretion on the 3rd day, 506 mg., is distinctly less than the normal endogenous excretion for a person weighing 98 kilos.

The subject, M-th, Table XLIII, received practically the full 20 mg. of uric acid per kilo, yet in this case also it is impossible to try to calculate any values corresponding to recovery of administered uric acid. On the day of injection (1,180 mg.), the total uric acid excretion was only 560 mg. This was followed by 785, 675, and 700 mg., for the following 3 days. Again "delayed excretion" in the sense of earlier investigators. Considerable fever, 102°F., but no joint pains developed in this subject during

TABLE XLII.
Showing Uncertain Recovery of Injected Uric Acid Probably Due to Excessive Variations in Uricolytic Process.
 Subject B-I. Age 41 years; weight 98 kilos. Diagnosis: gout.

Time.	Plasma.		Urine.				Remarks.	
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.			Total N per 24 hrs.
	mg.	mg.	Per hr.	cc.	Per hr.	Per 24 hrs.		
June-July, 1923.								Since June 17 on a purine-free low protein diet.
21							mg.	Average endogenous uric acid 366 mg.
22				810		357	4.1	
23				1,530		408	5.1	
24				1,180		348	4.3	
25				1,320		350	4.6	
11.18 a.m.		7.4	24.9	79	19			1,000 mg. uric acid intravenously.
11.21-24 a.m.								
11.34 a.m.	12.0	28.2						
12.12 p.m.	10.9	26.1						
2.20 "			86		38			
4.40 "			82		32			Uric acid recovered: 280 mg. 28 per cent.
4.52 "	9.8	25.5						
7.00 a.m.			39		24			
26	9.6	26.1		1,360		632	5.3	
27				750		455	3.9	
28	8.0	23.4		1,010		506	4.7	2.5 gm. atophan.
29				1,120		420	4.3	
30				2,070		427	4.4	
1	7.7	24.0		1,040		323	4.0	
2	6.5	25.8		1,080		636	4.1	
				1,260		354	4.2	

TABLE XLIII.
Sustained Excretion after Uric Acid Injection Indicates Extreme Recession of the Process of Destruction Rather Than Delayed Excretion.
 Subject Mr. M-th. Age 52 years; weight 64 kilos. Diagnosis: gout.

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		
			Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.	
	mg.	mg.	cc.	cc.	mg.	mg.	Total N per 24 hrs.
22	7.3	25.5					gm.
23				1,860		545	10.5
24				1,040		488	13.4
25				1,260		430	12.1
26				1,410		455	13.7
27				1,564		483	15.3
28	7.1	41.4		1,260		400	15.3
29				1,330		505	16.5
4				800		580	10.0
5				600		318	7.8
6			25		13		
11.22 a.m.	5.6	33.0					
11.23-25 a.m.							
11.40 a.m.	15.2	34.8					
1.10 p.m.	12.4	33.3					
1.14 "			37		36		
3.30 "			31		15		
5.35 "	11.1	41.1					
6.00 a.m.			56		29		
7	10.5	34.8		1,085		560	6.6
8				870		785	8.9
9	11.4	26.0		820		675	8.1
				660		700	8.4
</							

the night following the injection and lasted for 2 days. This fever must be considered as partly responsible for the increased uric acid excretion, and it is to be noted that on the morning of the 4th day, the plasma uric acid was 11.4 mg., whereas 6 hours after the injection it was 11.1 mg. The excessive excretion has therefore produced no sign of diminution in the circulating supply of uric acid.

It is easy to see how results such as those recorded in Table XLIII have led earlier writers to postulate gradual retention of uric acid during periods of no attacks, followed by elimination of the "stored" uric acid during and following attacks. As it happens, this patient, M-th, was treated with atophan for 3 days up to a short time (3 days) before the injection of uric acid, and in fact had not quite regained his usual level of circulating uric acid on the morning of the injection, so that, instead of carrying unusual amounts of "stored" uric acid, he probably had less than his usual amount. We conclude, therefore, that in this subject the fever inaugurated a change in some of the variable factors which determine the excretion of endogenous uric acid destruction or speed of excretion. Since the uric acid was rising instead of falling in the blood plasma, it seems reasonable to conclude that it was the uric acid-destroying process which receded. The fever may also have been responsible for some extra production of uric acid, but there was no increase in the creatinine excretion.

The remarkable change of level of uric acid excretion shown by M-th, Table XLIII, indicates that excessive (supernormal) excretion may indeed occur in gouty individuals, as has been found by previous observers. In ascribing such supernormal excretion to some other change than that of merely removing previously stored uric acid we have had in mind the unusual figures shown in Table XLIV.

Dr. S-n, the subject of this experiment, had not had an attack for nearly a year, and had none when examined, or since (3 months ago). His average endogenous uric acid excretion on a purine-free, low protein diet was 958 mg. The average normal excretion is about 7 mg. per kilo, and the excretion for S-n should have been about 600 mg. On a high protein, purine-free diet (eggs and cheese, etc.), Dr. S-n had an average endogenous uric acid

TABLE XLIV.
Showing Nearly Complete Recovery of Injected Uric Acid. Also Extraordinary Excretion of Endogenous Uric Acid.
 Subject Dr. S-n. Age 29 years; weight 87 kilos. Diagnosis: gout.

Time.	Plasma.		Urine.				Remarks.
	Uric acid per 100 cc.	Non- protein N per 100 cc.	Volume.		Uric acid.		
			Per hr.	Per 24 hrs.	Per hr.	Per 24 hrs.	
Jan. 1924.	mg.	mg.	cc.	cc.	mg.	gm.	
9							
10				1,085	900	6.9	
11	9.9	24.0		800	952	7.0	
12				950	1,040	6.5	
13				1,610	940	5.3	
11.06 a.m.	9.8	28.5	28				
11.08-11 a.m.					45		
11.14 a.m.	24.6	24.6					
11.29 "	21.6	24.9					
11.46 "			141		445		
12.32 p.m.	18.0	27.3					
12.36 "							
1.24 "			408		219		
5.08 "			200		183		
5.13 "			137		132		
12.09 a.m.	13.7	24.0	83		91		
9.00 "			18		34		
				1,919			
14	12.1	24.0		760	2,226	6.3	
15	11.0	21.6		720	1,230	5.5	
16				1,005	1,100	5.1	
17	10.4	19.2		740	940	5.3	
18	9.3				920	4.6	

excretion of 1,170 mg. It is certain that this gouty person regularly and continuously was excreting in the neighborhood of 1 gm. of endogenous uric acid per day. How much he produced or destroyed is, of course, impossible to say, but we are quite positive that his excretion did not represent previously stored uric acid. He had no tophi.

Dr. S-n weighed 87 kilos, and received intravenously 1,800 mg. of uric acid. The uric acid level in the plasma rose from 9.8 to 24.6 mg., a rise practically identical with the increases obtained from D-k and B-d, our first two normal subjects. No less than 70.4 per cent of the injected uric acid was eliminated during the first 21 hours. The total recovery amounted to 93.5 per cent, and might have been even greater, since there is room for choice as to the real average endogenous value. These figures indicate a speed of excretion at least equal to the best obtained from our normal subjects. They also indicate a lower intrinsic rate of uric acid destruction than that obtained in any normal subject. In making this statement we refer not only to the total recovery, but also to the high uric acid levels in the plasma—so different from those of H-w-d, Table XXVI, and also to the excessive endogenous uric acid.

It need scarcely be mentioned that in ascribing a subnormal speed of uric acid destruction to Dr. S-n, we are in no sense supporting the view of earlier writers that diminished destruction of uric acid is a general characteristic of the gouty. On the contrary, increased destruction is the predominant and practically inevitable characteristic. Diminished destruction goes only with increased elimination, which is exceptional, while the much more common subnormal excretions signify increased destruction.

In Table XLV are given the average endogenous uric acid excretions obtained from our different normal and gouty subjects. From the data given in this table it will be seen that only a single one of our nine gouty subjects has given normal values for the endogenous uric acid excretion. The normal values vary between 6.8 and 8.1 mg. per day and per kilo of body weight. Six of the gouty have given corresponding values of from 3.6 to 7.0 mg., and the other three have varied between 9.1 and 11 mg. The variations found in normal subjects are thus very small in comparison with the variations occurring among

the gouty. The differences among normal persons, as well as daily variations in any one individual, can be best explained just as we have explained the different excretions of injected uric acid, namely on the basis of normal variations in the speed of excretion and the speed of destruction—the last named process being more or less modified by the level of uric acid circulating in the plasma.

TABLE XLV.

Showing Abnormal Excretions of Endogenous Uric Acid in Gout. Purine-Free Low Protein Diet.

Subject.	Table.	Weight.	Uric acid.		Remarks.
			Average daily excretion.	Daily excretion per kilo of body weight.	
		<i>kg.</i>	<i>mg.</i>	<i>mg.</i>	
H. B-d.	XXIV	88	605	6.9	Normal.
D-k.	XXV	67	461	7.0	"
H-w-d.	XXVI	62	504	8.1	"
B-n.	XXVII	70	489	7.2	"
W-n.	XXVIII	62	425	6.8	"
B-ck.	XXIX	77	570	7.2	"
K-z.	XXXVI	81	350	4.3	Gout.
B-th.	XXXVIII	50	352	7.0	"
Br-k.	XXXIX	70	355	5.1	"
G-er.	XL	50	244	4.9	"
S-ds.	XLI	70	425	6.1	"
B-l.	XLII	98	366	3.6	"
M-th.	XLIII	64	687	10.8	"
S-n	XLIV	87	958	11.0	"
Gl-y.	XLVI	97	889	9.1	"

To us it seems premature and unprofitable to try to explain the abnormal variations among the gouty except on the basis of abnormal deviations in these two known variables—speed of excretion and speed of destruction. It must be recognized, of course, that in explaining the abnormally high uric acid excretions in gout, on the basis of diminished destruction, we are compelled to assume that the endogenous uric acid production in man is much greater than is indicated by normal uric acid excretions, but this seems to us a more probable assumption than

the only other available alternative, namely abnormal production of uric acid in some gouty subjects.

Theoretically one cannot definitely exclude the occurrence of some synthesis of uric acid in mammals, as was intimated in connection with the enormous uric acid excretions encountered in the Dalmatian dog, Table XVII. If such synthesis ever does occur, it represents probably a rare and relatively slight reversion to an older type of metabolism. The present state of knowledge does not warrant the introduction of this concept as a feature of the uric acid metabolism in gout.

The concept of possible uric acid synthesis, in so far as it is ever revived, must necessarily involve renewed studies of the allantoin problem, for it is by no means excluded that the allantoin excretion of present day mammals (absent in man) may in fact represent unfinished synthesis of uric acid, more than it represents destruction of endogenous uric acid.

The outstanding feature in gout is the high level of circulating uric acid. Of about fifteen cases examined, only one failed to show abnormally high values. [That subject was under treatment for syphilis and was not available for detailed investigation. He was running a constant fever (100–101°F. in the afternoon) of unrevealed origin.] This general characteristic of gout must surely be interpreted as due to a pathological deterioration of a process which normally functions much less effectively in man than in other mammals, namely the power to excrete uric acid. But from our experiments with animals it has become reasonably certain that the function of the kidneys, at least in relation to uric acid, is made up of two more or less distinct and different processes—preliminary absorption and concentration within the kidneys, followed by the second process—excretion. The abnormal inferiority of the gouty kidney, therefore, can be either an inferior power to excrete uric acid which has accumulated in the kidneys, or it can be an abnormal lack of responsiveness to the levels of circulating uric acid, on the part of the uric acid-absorbing power. We believe that it is the latter function which is poor in man and abnormally poor in the gouty. In this connection we would refer to the behavior of Dr. S-n, Table XLIV. The speed of uric acid excretion exhibited by this subject was equal to that of the very best found among our normal subjects.

but that rapid excretion occurred on the basis of a very high level of circulating uric acid. If the *uric acid-absorbing process* of Dr. S-n's kidneys had not been subnormal, their excellent power to excrete uric acid should have been quite capable of maintaining a normal level in the plasma.

Gl-y, Table XLVI, our last subject, exhibited almost the same degree of high endogenous uric acid excretion as Dr. S-n, and in this case we expected to obtain from the uric acid injection about the same rapid excretion and high recovery. The reason why we did not obtain this result is, however, quite clear. The injection of about 20 mg. of uric acid per kilo in this case had substantially the same effect as we have often obtained from injections of larger quantities (100 mg.) in dogs, and also in goats. The patient had had some fever while the attack was acute and his non-protein nitrogen had risen from 30.3 to 46.8 mg. during the 5 days intervening between the taking of the two preliminary bloods. The rapid and excessive increase in the non-protein nitrogen after the uric acid injection is, however, of an entirely different order. The non-protein nitrogen rose in 2 hours to 70.8 mg. and the peak was reached 2 days later, at 87.6 mg. There can be no doubt about the fact that the kidneys in this case became overcharged with uric acid and almost lost the power to excrete anything. The subject was kept for a long time and his non-protein nitrogen gradually returned to the original normal level. In our first gout subject, K-z, Table XXXVII, we obtained a transient rise of the non-protein nitrogen from 34.8 to 60.6 mg., but in that case complete restoration of the circulating nitrogen level was restored within 24 hours. We have obtained no similar result from any normal subject.

Much has been written from a clinical point of view about the frequent development of a more general nephritic involvement among gouty subjects. In the course of our study of gout, we have observed and produced small, but unmistakably abnormal, levels and increases in the non-protein nitrogen on so many occasions (by means of high protein diets) as to compel the conclusion that the kidneys in the gouty are nearly always inferior to the kidneys of normal persons. This weakness is probably not a late and secondary development, but rather an accompaniment of the deterioration responsible for the high uric acid

Showing Excessive Endogenous Uric Acid and Yet No Recovery of Injected Uric Acid—Because of Temporary Injury to Kidney.
 Subject Mr. G1-y. Age 56 years; weight 97 kilos. Diagnosis: gout.

Time.	Plasma.		Urine.						Remarks.			
	Uric acid per 100 cc.		Non- protein N per 100 cc.		Volume.		Uric acid.			Total N per 24 hrs.	Creatinine per 24 hrs.	
	mg.	mg.	per hr.	Per 24 hrs.	Per hr.	mg.	Per 24 hrs.	mg.				
Mar., 1924.												Since Mar. 5 on very low purine-free diet; on Mar. 9 increased to 50 gm. protein and 1,200 calories.
5												
7												
8												
9												
10												
11												
10.39 a.m.												
10.45-48 a.m.												
11.03 a.m.												
11.50 "												
12.33 p.m.												
3.50 "												
4.00 "												
6.05 a.m.												
12												
13												
14												
15												
16												
17												
18												

Fluid from left knee joint: 8.9 mg. uric acid, 36.9 mg. non-protein N, 5.3 gm. protein per 100 cc.

1,800 mg. uric acid intravenously.

Chill.

Fluid from left joint: 14.8 mg. uric acid, 73.3 mg. non-protein N, 5.25 gm. protein per 100 cc.

levels. This condition limits in a measure the merit of high protein diets as a means of reducing the level of circulating uric acid in gouty individuals. The effect of such diets certainly should be always checked up by means of occasional determinations of the non-protein nitrogen as well as the uric acid.

In no case has the urate injection produced or aggravated the gouty attacks.

VII.

RÉSUMÉ.

1. When uric acid is injected into the blood stream of animals (dogs, cats, rabbits, goats), the kidneys immediately take up very much uric acid. The kidneys at the same time become edematous and greatly enlarged. Up to fully 0.2 per cent of uric acid may be temporarily stored in the kidneys.

2. The uric acid which cannot find lodgement in the kidneys remains to a large extent in the circulating blood until it is destroyed; in other words, all the other tissues seem to be more or less completely impermeable to soluble urates.

3. The specialized uric acid-absorbing power of the kidney involves the whole organ and (in the dog) does not represent merely arrested excretion, since only an insignificant fraction is excreted. In this animal (rapid destruction), the rest goes back into the blood as soon as the uric acid in the blood has sunk to sufficiently low levels.

The excretory function of the kidney may be temporarily diminished up to full inhibition as a consequence of the injurious effect of the uric acid (tubular injury); the kidney in such a case (in the goat—slow destruction) remaining loaded with uric acid for hours, even after the blood level has become quite low.

4. The destruction of uric acid in the dog, and presumably in all other animals, seems to take place within the circulating blood, though it is possible that some is destroyed in the kidneys. This conclusion is based partly on the fact that the uric acid cannot enter the other tissues and organs, including the liver, and partly on the fact that no demonstrable disappearance of uric acid is due merely to the passage of the blood through tissues.

5. The destruction of uric acid within the circulating blood proceeds with extreme velocity during and immediately after

an injection (100 mg. per kilo), but soon slows down to a measurable speed, and the speed continues to diminish as the concentration of the circulating uric acid continues to sink. The complete destruction of the injected uric acid in dogs is accomplished in about 2 hours. Not less than 70 per cent is destroyed within the first 10 minutes. The speed of the destruction is different in different dogs, but is never irregular, and the predominant factor is always the concentration of the circulating uric acid.

6. The uric acid destruction stops the instant the blood is removed from the living animal. Some unknown essential factor, therefore, must be contributed by the tissues, or by some tissue—possibly the liver. The unknown oxidizing agency is apparently used up as rapidly as it is poured into the blood. This agency, therefore, can scarcely be an enzyme.

7. The specialized uric acid-absorbing power of the kidney and the impermeability of living muscle to uric acid can be most easily shown by uric acid determinations in birds. Their kidneys contain normally about 0.1 per cent of uric acid while the blood contains about 0.007 per cent and the muscles 0.001 to 0.002 per cent.

8. The speed of uric acid destruction in herbivorous animals (goats, rabbits) is less than one-tenth as rapid as that found in the dog. The destruction is slower in the goat than in the Dalmatian hound.

9. Since the blood of herbivorous animals carries very low levels of uric acid and since the speed of destruction is mainly determined by the concentration in the blood, herbivorous animals probably destroy very little endogenous uric acid.

10. Intravenous injections of uric acid (20 mg. per kilo in the form of lithium urate) into normal men are followed by excretions of from 30 to 90 per cent. The destruction in man, therefore, amounts to from 10 to 70 per cent, the average being about 50 per cent.

11. The losses (destructions) in man are variable because the two factors involved—speed of destruction and speed of excretion—are subject to independent variations.

12. The excretion period in man lasts from 1 to 4 days. The duration is mainly determined by the speed of destruction.

13. There is no tangible reason for assuming that the distribution of injected uric acid is different in man from the distribution found in animals. It usually takes from 2 to 4 days for the circulating uric acid to reach its normal level after an injection of 20 mg. per kilo. The initial increase varies between 5 and 15 mg. per 100 cc. of blood plasma.

14. The unique and characteristic high levels of uric acid in normal human blood are due to a lack of responsiveness on the part of the human kidney. This lack of sensitiveness is probably a feature of the uric acid-absorbing power, rather than of the power to excrete any uric acid that has been absorbed. Each individual tends to carry his own level of circulating uric acid.

15. High protein diets increase the responsiveness of the kidneys and thus lower the level of circulating uric acid. Because of these lower levels less uric acid is destroyed and this is the reason why more endogenous uric acid is excreted on high protein than on low protein diets.

16. The characteristic lack of responsiveness on the part of the human kidney is exaggerated in gout and this is the main or only reason why the gouty carry abnormally high levels of circulating uric acid.

17. The uric acid-destroying process in the gouty is intrinsically about the same as in normal persons but is subject to wider variations. The high levels of circulating uric acid in the gouty automatically result in more extensive destruction and therefore in diminished excretion (unless the power of destruction is very small).

18. The distribution of injected uric acid is substantially the same in gouty subjects as in normal persons. There is no reason to believe that the muscles of gouty individuals contain much more uric acid than the muscles of normal subjects. These conclusions are based on the finding of the same increases in the plasma as in normal persons after uric acid injections.

19. Intravenous uric acid injections (20 mg. per kilo) do not produce attacks in the gouty, but may produce enough temporary injury to the kidneys to result in a transient retention of other nitrogenous waste products than uric acid (increased non-protein nitrogen and urea of blood). In normal persons no such retentions have been encountered.

20. While urates do not diffuse into living cells of ordinary tissues (muscle), the possibility remains that cartilage and connective tissue, like dead animal tissues, may permit a slow passage of urates into the localities where urates are found in the gouty.

The authors are indebted to Dr. Joseph B. Howland, Superintendent of the Peter Bent Brigham Hospital, for granting us the free use of the hospital supplies and facilities for the long continued constant diet experiments with normal persons. We are also indebted to the Hospital dietitians, Mrs. Octavia Hall Smilie and Miss Amalia Lautz for unfailing willingness to provide the special diets for all our subjects. For the conscientious and successful collection of the urines from our gouty subjects we must express our thanks to the nurses of ward F. M.

For much and resourceful help in the laboratory during the latter part of the work we owe thanks to Miss Hazel Hunt.

A SYSTEM OF BLOOD ANALYSIS.

SUPPLEMENT V.

IMPROVEMENTS IN THE QUALITY AND METHOD OF PREPARING THE URIC ACID REAGENT.

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(Received for publication, April 1, 1924.)

The problem of making the uric acid reagent of Folin and Denis as specific as possible for uric acid has become more important since Benedict¹ brought out the fact that substantially correct uric acid values for blood filtrates can be obtained without any preliminary precipitation of the uric acid. If uric acid reagents made from different samples of sodium tungstate are compared with reference to the amount of color which they give with blood filtrates obtained from sheep or dogs—filtrates which contain only traces of uric acid—it will be found that the reagents are not equivalent. Some will give distinctly more color than others. Similar differences are obtained if the reaction is applied to 0.2 mg. of resorcinol added to 5 cc. of water.

The different results given by different samples of the uric acid reagent could obviously be due to variations in the amounts of molybdenum which may be present in the available brands of sodium tungstate. In their first paper on the preparation of the uric acid reagent, Folin and Denis stated that the sodium tungstate used must, of course, be free from molybdenum. This statement was based on their finding that when mixtures of tungstate and molybdates were used, the reagents became more sensitive to reducing substances, including the phenols. But molybdenum is always present in tungsten ores and there is very little

¹ Benedict, S. R., *J. Biol. Chem.*, 1922, li, 187; liv, 233. Folin, O., *J. Biol. Chem.*, 1922, liv, 160.

reason to believe that the molybdenum has been completely removed from the sodium tungstates available from dealers or manufacturers.

We have accordingly examined all the brands of sodium tungstate available in this department or obtainable from dealers. All, including the c. p. grades, contain appreciable quantities of molybdenum and some contain much larger amounts than others. One particularly bad sample had on the container the trade mark "Folin's purity" in addition to the customary c. p.

In this connection attention might be called to the test which we have found to be the most satisfactory for the detection of small quantities of molybdenum in tungstates. The test,² first suggested by Siewert, in 1864, is carried out as follows:

Dissolve about 1 gm. of the sodium tungstate in 5 to 10 cc. of water. The reaction should be alkaline to litmus. Add a pinch, 0.2 gm., of solid potassium xanthate and shake until it has dissolved. Add to the solution so obtained 20 per cent sulfuric acid, 1 drop at a time, with shaking. The shaking should be continued after each drop until the curdy tungstic acid precipitate which is produced has dissolved. If molybdenum is present it will be noted that the white turbidity which appears as the acid is added takes on a pinkish tint in the course of a few seconds. As the solution becomes definitely acid the entire solution takes on a color varying in shade from pink to a deep plum, which persists after shaking. Continue the addition of acid, with shaking, until there is no further increase in the depth of the color. Add about 2 cc. of chloroform, shake vigorously, and allow to separate into two layers. The molybdenum xanthate is taken up by the chloroform layer which will become more or less reddish in color, depending on how much molybdenum is present. This test furnishes a rapid method for comparing the molybdenum content of different tungstates.

Preparation of Potassium Xanthate.—In a 200 cc. Erlenmeyer flask place 100 cc. of absolute ethyl alcohol and 10 gm. of potassium hydroxide which has been broken into small lumps. Heat the mixture on a steam bath, shaking frequently, until nearly all the

² This reaction has recently been made the basis of a quantitative gravimetric method for molybdenum in tungsten compounds by Hall (Hall, D., *J. Am. Chem. Soc.*, 1922, xlv, 1462).

solid dissolves. Filter the solution into another Erlenmeyer flask to remove the insoluble material (carbonate). Then, while the solution is continuously agitated, add, a few drops at a time, 10 cc. of carbon disulfide. Set the solution aside to cool and crystallize. The potassium xanthate separates out as a fluffy mass which completely fills the solution. Transfer the precipitate to a Büchner porcelain funnel and filter with the aid of suction. Press the precipitate down on the funnel with a spatula to facilitate complete removal of the mother liquor. Wash the precipitate once with a small portion of absolute alcohol, and once with ether. The potassium xanthate has a faint yellowish tint and a very characteristic odor. The yield is about 12 gm. It can be preserved in a stoppered bottle for a long time.

By means of this test all the sodium tungstates now in the market give positive reactions for molybdenum. The amount of color, which uric acid reagents made from these tungstates give with 0.2 mg. of resorcinol, is approximately proportional to the molybdenum content. The molybdenum cannot be removed by means of ordinary recrystallizations (Smith and Exner³). In fact it is extremely difficult to remove the last traces of molybdenum by any process and we have wasted much time trying to find a suitable method for the preparation of sodium tungstate absolutely free from molybdenum. We have obtained it in small quantities quite free from molybdenum. One sample was examined for us by Professor F. A. Saunders, of the Jefferson Physical Laboratory, Harvard College. He photographed its spectrum and found no trace of molybdenum. But the purification process used could not readily be applied on a sufficiently large scale and we therefore omit further discussion of it here.

We have, however, succeeded in devising a comparatively simple and practical method for preparing the uric acid reagent almost absolutely free from molybdenum. The process depends on the fact that the molybdenum is precipitated as sulfide by hydrogen sulfide in the presence of phosphoric acid.

Preparation of the Improved Uric Acid Reagent.—Transfer 100 gm. of normal sodium tungstate, $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, and 160 cc. of distilled water to a 500 cc. flask. Add 50 cc. of 85 per cent phos-

³ Smith, E. F., and Exner, F. F., *Proc. Am. Phil. Soc.*, 1904, xliii, 123.

phoric acid, a few cubic centimeters at a time, with cooling under running water so as to prevent any appreciable rise in temperature. Connect the flask with a wash bottle leading to a Kipp's hydrogen sulfide generator as indicated in Fig. 1. *A* is an ordinary gas wash bottle half filled with water. *B* is the flask containing the mixture of sodium tungstate and phosphoric acid. *B* is equipped with a 2-hole rubber stopper fitted with an inlet tube *C* and an outlet tube *D*. The rubber stoppers in both *A* and *B* must fit so as to be gas-tight. To *D* is attached a short section of flexible rubber tubing

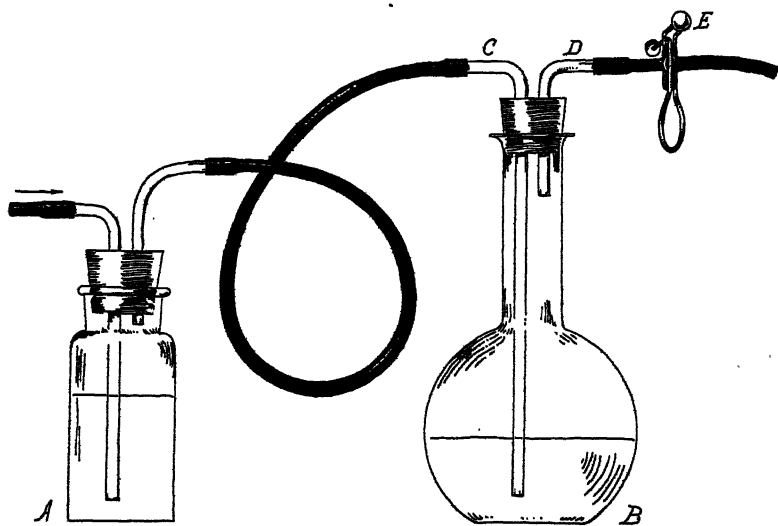


FIG. 1.

which is closed by the pinch-clamp *E*. The inlet tube of *A* is connected with the outlet of a Kipp generator furnishing hydrogen sulfide.

Open the stop-cock of the generator and then open the pinch-clamp *E* momentarily, and close again, thus allowing the hydrogen sulfide to bubble through the solution. Repeat the opening and closing of the pinch-clamp six or seven times in order to remove the air and fill the flask with hydrogen sulfide. Then close the pinch-cock (*E*), but leave open the stop-cock which admits the hydrogen sulfide to the apparatus. When connected in this way, hydrogen sulfide is produced by the Kipp generator only as it is

absorbed by the solution. The action of the hydrogen sulfide is continued overnight for the molybdenum sulfide precipitates only very slowly. The solution in flask *B* will gradually take on a deep blue color.

The next morning disconnect the apparatus and filter the solution in *B* into a 500 cc. flask. Avoid transferring the precipitate to the filter paper until most of the liquid has passed through. Allow the precipitate to drain completely, but do not wash it. Insert a 10 cm. funnel, and in this funnel place a 200 cc. flask, filled with cold water, and boil the contents of the flask gently, but continuously, over a micro burner, for 1 hour. The funnel and flask merely act as a convenient condenser. The boiling must not greatly reduce the total volume of the solution.

Filter the hot solution from the additional small quantity of molybdenum sulfide which comes out during the boiling. This time, washing is permissible. Wash with small quantities of water until most of the blue color has been removed from the filter.

To remove the blue color from the filtrate, heat the solution to boiling, remove the flame, and decolorize by the addition of bromine and shaking vigorously. Add only 2 to 3 drops at a time. A clear, light yellow solution should be obtained when sufficient bromine has been added. Boil the solution gently for 10 minutes to remove any excess of bromine and cool.

Meanwhile transfer to a liter beaker 25 gm. of lithium carbonate and add first 50 cc. of 85 per cent phosphoric acid and then 200 cc. of water. Boil off the carbon dioxide and cool.

Mix these two solutions and dilute to 1 liter.

The uric acid reagent, obtained in the process described above, gives only an extremely faint bluish tint with 0.2 mg. of resorcinol.

For ordinary work, such as the determination of uric acid in the silver lactate precipitate obtained from human urine or for ordinary clinical determinations of the uric acid content of human blood, it is scarcely necessary to remove the molybdenum from the uric acid reagent, because the color given by resorcinol is always very weak in comparison with the color obtained from uric acid. Errors up to 1 mg. per 100 cc. of blood may occur, however, as a result of using a reagent which contains large traces of molybdenum.

In connection with this research and while we were still hoping to find some brand of sodium tungstate free from molybdenum we worked out certain improvements in the ordinary process for making the uric acid reagent from (a) normal sodium tungstate, (b) sodium paratungstate, and (c) ordinary phosphotungstic acid.

Preparation of Uric Acid Reagent from Normal Sodium Tungstate.—Transfer 50 cc. of 85 per cent phosphoric acid and 160 cc. of water to a 500 cc. Pyrex flask. Heat nearly to boiling and then add 100 gm. of sodium tungstate. The mixture begins to boil from the heat of the reaction. Boil *gently* but continuously over a micro burner for 1 hour, using a 10 cm. funnel and a 200 cc. Florence flask filled with cold water, as a condenser.

Transfer 25 gm. of lithium carbonate to a liter breaker. Add 50 cc. of 85 per cent phosphoric acid and 200 cc. of water. Boil off the carbon dioxide and cool. Mix the two solutions and dilute to 1 liter.

Preparation of the Uric Acid Reagent from Sodium Paratungstate.—Transfer 40 cc. of 85 per cent phosphoric acid and 80 cc. of water to a 200 cc. Pyrex Florence flask. Heat nearly to boiling and then add 90 gm. of sodium paratungstate. Heat to boiling and continue the boiling for 1 hour after the tungstate has dissolved. Paratungstate dissolves more slowly than the normal tungstate and generates no heat. Use the same condenser during the boiling as is described in the preceding method and do not boil so fast as to produce much concentration.

Dissolve 25 gm. of lithium carbonate in a liter beaker with 50 cc. of phosphoric acid and 200 cc. of water. Boil, cool, mix the two solutions, and dilute to 1 liter.

Preparation of the Uric Acid Reagent from Ordinary Phosphotungstic Acid.—Transfer 10 gm. of lithium carbonate, 20 cc. of 85 per cent phosphoric acid, and 80 cc. of water to a 300 cc. Pyrex Florence flask. Heat until nearly the whole of the carbonate has dissolved. Then add 80 gm. of ordinary 24-phosphotungstic acid and continue the boiling gently for 1 hour with the usual funnel-flask condenser to prevent undue evaporation.

To a liter beaker add 15 gm. of lithium carbonate, 65 cc. of phosphoric acid, and about 200 cc. of water. Boil, cool, mix the two solutions, and dilute to 1 liter.

All the reagents are substantially identical. With 1 cc. of the reagent and 2 cc. of 15 per cent sodium cyanide they give the maximum color for the uric acid content of 5 cc. of blood filtrate, provided that the uric acid content of the blood does not exceed 8 mg. per 100 cc. and provided further that the cyanide used gives no blank. We have lately obtained, in the open market, a sodium cyanide (Roessler and Hasslacher), freshly prepared solutions of which give absolutely no blank.

It will be noted that in each case the required lithium has been incorporated in the reagent. The reagents give a white turbidity with the blood filtrates in the cold, but this turbidity disappears during the subsequent heating on the water bath, and does not return.

100

LEAD STUDIES.

IX. THE SOLUBILITY OF VARIOUS LEAD COMPOUNDS IN BLOOD SERUM.

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Knowledge of the solubility of lead and its compounds in serum or body fluids is important in understanding not only how lead is carried in the blood stream but also how it is absorbed from the mucous membranes and respiratory tract. In industry the most dangerous lead trades have proved to be those in which dust is inhaled; and experiments have shown (1) that poisoning develops very readily after inhalation of lead or certain lead compounds. Since the toxic symptoms are not caused by the local action of lead on the mucous membranes and in the respiratory tract, but by its action on tissues far distant from these, the mechanism of its entrance into the organism and its transportation in the circulation is of fundamental interest. Phagocytosis and direct absorption of dissolved lead compounds, as well as diffusion from particles in contact with the moist epithelial cells of the alveoli, must play some rôle in this mechanism. The purpose of this investigation was to seek more information regarding one of these factors; *i.e.*, to determine whether the solubility of lead compounds in the fluids of the tissues is different from that in pure water. For this purpose blood serum was selected as a representative medium.

EXPERIMENTAL PROCEDURE.

Materials.—Fresh horse serum, obtained through the courtesy of Dr. Benjamin White of the Massachusetts State Vaccine Laboratory, was used for these experiments. Pure finely granular “test-lead” (Baker and Adamson) was employed. Lead sulfate, chromate, and carbonate were prepared by precipitation from pure lead ace-

tate, and were repeatedly washed to remove soluble impurities. *Lead oxide* was derived from this pure lead carbonate by ignition in an electric muffle furnace. This oxide was free from soluble salts and lead carbonate.

Method.—An excess of the dry solid lead or lead salt was added in 250 cc. bottles to fresh horse serum containing a small amount of powdered thymol as preservative. The bottles were then securely sealed and rotated mechanically, end over end, at a rate of 38 R. P. M. for 24 hours in an electrically heated thermostat which held the temperature constant within 0.1° at 25°C . At the end of this period the mixtures were allowed to stand in order to separate the solid phase. Finally the last traces of solid were removed by repeated centrifugalization. Portions of the clear solution were analyzed in triplicate for lead by ashing, converting the lead to chromate, and titrating it in this form with 0.005 N sodium thiosulfate (2).

The solubility data for lead and for the compounds investigated here are collected in the following table:

No.	Substance.	Solubility in serum at 25°C .	Solubility in H_2O .	
		gm. per l.	gm. per l.	
1	PbCO_3	0.0333	0.0017	at 18° (3)
2	PbSO_4	0.0437	0.044	" 24.95° (4)
3	PbCrO_4		0.00001	" 25° (5)
4	PbO	1.1520	0.0171	" 20° (3)
5	Pb	0.578		

Effect of Carbon Dioxide.—The effect of carbon dioxide on the solubility of these substances was determined by saturating the fresh serum suspension of the solid with carbon dioxide gas at 25° and determining the solubility at 25° as above. The following values were obtained:

No.	Substance.	Solubility in CO_2 -saturated serum.
		gm. per l.
1	PbCO_3	0.0125
2	PbSO_4	0.0300
3	PbO	1.1600
4	Pb	0.1930

These values, while somewhat below normal, do not indicate a marked change in the solubility except in the case of metallic lead. This exception probably depends upon the fact that metallic lead reacts with the CO_2 so that the individual granules become coated with a film of lead carbonate which prevents further oxidation. It has been found in this laboratory that the tendency of lead to oxidize in contact with pure water is greatly reduced by the presence of a small amount of CO_2 . Thus, 100 cc. of pure water, which is shaken with metallic lead for 60 seconds, oxidize 117.0 mg. of Pb; whereas 100 cc. of pure water, 25 per cent saturated with CO_2 and shaken with lead in the presence of air for the same length of time, oxidize only 10.3 mg. of Pb. It is quite probable that carbon dioxide hinders oxidation by forming a protective coating of lead carbonate on the lead granules. The fact that CO_2 has no apparent effect on the solubility of PbO in serum is not of great significance as the CO_2 appears to be probably rapidly fixed by the PbO .

The solubility of lead oxide in serum is the most striking of the results obtained. While its solubility in pure water is only 17 mg. in serum it is greatly increased and amounts to 1.15 gm. This solution is alkaline to phenolphthalein. Doubtless as lead hydroxide is formed it is rapidly removed by protein, which combines with it to form an alkali metaprotein. It would be expected, therefore, that lead fume or lead oxide, when carried into the respiratory tract, would find an easy mode of entrance into the circulatory system.

The experiments with lead chromate indicate that its solubility is no greater in serum than in pure water. Some reduction of the chromate by serum would be expected in the course of 24 hours, for it is readily reduced by a number of organic substances. This is interesting in view of the fact that insufflation experiments have indicated a fairly rapid removal of lead chromate from the lungs (1). However, although the serum itself exerts no reducing effect, the enormous avidity of the tissues for oxygen, as shown by Ehrlich's experiment with methylene blue, is well known. It would not be surprising, therefore, if so powerful an oxidizing substance as lead chromate would suffer reduction within the body.

Experiments with precipitated lead sulfide are unsatisfactory because it is difficult to separate the solid phase by centrifugalization. Furthermore, lead sulfide is sensitive to oxidation. Figures finally have been obtained which correspond more nearly with those obtained with lead sulfate and have been ruled out on this account. Lead carbonate is somewhat more soluble in serum than in pure water. The solubility is reduced slightly by CO_2 .

CONCLUSIONS.

The results obtained indicate no great increase in the solubility of lead salts in serum as compared with that in pure water. The marked solubility of lead oxide and metallic lead is, however, significant for workers in the lead industry as a probable factor in the poisoning which develops rapidly after exposure to lead fume and lead oxide dust.

Legge and Goadby (6) show that the proportion of those suffering from lead encephalopathy is higher in the manufacture of lead oxides than in any other lead industry. It is quite possible that the ready solubility of these compounds allows lead to enter the system more quickly than it can be eliminated or deposited in the bones as relatively harmless lead phosphate and that the large quantities circulating tend to produce severe lead intoxication.

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LEAD STUDIES.

XI. A RAPID METHOD OF ANALYZING URINE FOR LEAD.

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(Received for publication, April 11, 1924.)

There is great clinical need for an accurate and rapid test for lead in urine because lead may be found in the urine only after it has been absorbed. The older and more obvious methods of analysis have so far proved very unsatisfactory. It is impossible to precipitate lead from urine quantitatively by the ordinary precipitants for lead because the insoluble lead salts become highly dispersed and remain in colloidal solution. A second objection to this method is the mechanical difficulty of filtration, which is slow and uncertain. Equally unsatisfactory is the precipitation of lead on magnesium ribbon (1) and by direct electrolysis (2). To overcome these difficulties urine must be evaporated to dryness and completely ashed before the quantitative test for lead is made. The slow oxidation of the carbonaceous residue, in the presence of a large quantity of fused salts, as well as the repeated extraction of the residue with water and acid, and the necessary reashing, renders such a process very time-consuming. Attempts to precipitate excreted lead by bubbling hydrogen sulfide gas directly into urine have resulted in failure because the lead is probably present in the form of highly dispersed lead phosphate in the presence of dissolved phosphates, and is, therefore, more insoluble than lead sulfide. A new method of separating lead salts from urine has therefore been devised.

Attempts to simplify the usual procedure by separating lead by adsorption on charcoal, or by chemical precipitation on bone meal or permutit were not encouraging. It was found possible, however, to precipitate lead from urine quantitatively by *entrainment* with the earthy phosphates. Precipitation by entrainment is of great value in separating minute amounts of a substance from

a large volume of fluid—particularly when addition of a precipitant causes the formation of a colloidal precipitate which may not settle out completely for several days. The simultaneous coagulation of such a salt and of a large volume of some other salt bearing the same anion allows complete precipitation of both; *i.e.*, entrainment of the smaller precipitate. By this method Meillère detected minute amounts of mercury in water by adding

TABLE I.

No.	Lead added.	Lead recovered.	Error.
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	1.00	0.94	-0.06
2	1.00	1.04	+0.04
3	1.00	1.07	+0.07
4	1.00	1.02	+0.02
5	1.00	1.04	+0.04
6	1.00	1.04	+0.04
7	1.00	1.07	+0.07
8	1.00	1.02	+0.02
9	1.00	1.06	+0.06
10	1.00	1.00	0.00
11	1.00	1.00	0.00
12	1.00	1.07	+0.07
13	1.00	0.99	-0.01
14	1.00	1.04	+0.04
15	1.00	0.98	-0.02
16	1.00	1.03	+0.03
17	1.00	1.06	+0.06
18	1.00	1.01	+0.01
19	1.00	0.96	-0.04
Average.....			±0.037

a soluble copper salt and subsequent precipitation of both metals with hydrogen sulfide (3). The same principle has been employed in separating lead from urine.

If fresh or well preserved urine is made strongly ammoniacal, the alkaline earth phosphates precipitate in a gelatinous mass so completely that the clear liquid above can be decanted. A series of experiments was carried out with urine to which known amounts of lead had been added before the addition of ammonia. In each case the volume of urine used was 1 liter and the quantity of

lead 1 mg., or 1 part in one million. The precipitates filtered out quite rapidly on a Büchner filter by suction and were ashed before quantitative measurements were made. Because the precipitate contains relatively little inorganic salt, as compared with the residues obtained by evaporation of urine, the lead is the more easily separated. The data obtained by volumetric determination of the lead as chromate (4) are summarized in Table I. These results show that the recovery of lead from the precipitate was satisfactory, particularly since no end-point correction (about 0.03 mg. per drop) was used.

TABLE II.

No.	Volume of urine.	Lead in precipitate.	Lead in filtrate.
	cc.	mg.	mg.
1	3,075	0.07	Negative.
2	3,500	0.05	"
3	15,000	0.38	"
4	4,020	0.08	"
5	5,540	0.05	"
6	3,800	0.04	Positive (microchemically).
7	4,420	Negative.	Negative.
8	4,270	0.28	"
9	3,500	0.19	"
10	3,500	0.20	"
11	3,150	Negative.	"
12	4,570	0.23	"
13	4,730	0.73	"

This method of analysis was also applied to the urine of patients suffering from lead poisoning. Frankel (5) had not been able to detect excreted lead electrolytically, while he had been able by the same method to demonstrate the presence of lead added to urine, and it was therefore felt desirable to determine whether the entrainment method involved this difficulty. Both the filtrates and the phosphate precipitates were analyzed separately in this series of experiments to ascertain whether any lead had escaped precipitation. When there was too little lead present in either to allow quantitative analysis, a microchemical test was used (6). The results, which are collected in Table II, show that excreted lead can be removed by precipitation of the phosphates of urine with ammonia and that the filtrate is lead-free.

In using this entrainment method a few precautions are necessary to insure success. The urine must be either freshly collected or well preserved with powdered thymol, because the crystalline phosphates which form when urine is allowed to become ammoniacal on standing do not completely remove the lead. Experiments have shown that if this precipitate is dissolved in mineral acid, and the urine then rendered ammoniacal, less than 50 per cent of the lead is present in the precipitate. Boiling ammoniacal urine to increase the rate of settling of phosphates prevents complete recovery of lead. Heating should therefore be avoided. The most convenient method of obtaining the precipitate is to add an excess of ammonium hydroxide to the urine and to let the mixture stand overnight at room temperature in a large Erlenmeyer flask. Although the precipitate settles out within 2 to 3 hours, it is desirable to allow this longer interval, as the precipitate is then quite compact and may be separated rapidly by suction filtration.

SUMMARY.

An entrainment reaction has been applied to the precipitation of lead from urine. Lead is quantitatively precipitated with the alkaline earth phosphates when ammonium hydroxide is added. This precipitate can be collected, ashed, and the amount of lead present determined volumetrically with greater facility than by the usual method of evaporating the urine to dryness and ashing the residue.

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THE INFLUENCE OF INSULIN ON THE GLUCOSE-FERMENTING ACTION OF *BACILLUS COLI*.

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(Received for publication, May 3, 1924.)

INTRODUCTION.

Because of the widely distributed occurrence of "insulin" and analogous bodies in the less highly developed organisms such as shell-fish and plants¹ as well as in the animal body, it seemed possible that the carbohydrate metabolism of bacteria might be influenced by insulin.

For a number of reasons the glucose-splitting bacteria *Bacillus coli* seemed to be especially fitted for this study. The glucose-splitting reaction of *Bacillus coli* takes place at body temperature; the main end-products are carbon dioxide and water; the metabolism of *Bacillus coli* is sensitive to added substances; the material lends itself to experimental control; etc.

EXPERIMENTAL.

A 12 to 18 hour old *Bacillus coli* culture² was diluted about twenty times with neutral meat broth containing peptone and about 1 per cent glucose at 37.5°C. From this *Bacillus coli* broth mixture, 100 cc. portions were added to flasks containing various amounts of the commercial insulin preparation "Iletin" and enough water to make the final volume 102.5 cc., plugged with cotton, and incubated at 37.5°C. in a constant temperature water bath. At definite time intervals 5 cc. portions were removed from the different mixtures, the proteins precipitated by the Folin and

¹ No specific insulin references are given in this short paper. The reader is referred instead to the very complete review of the literature given in Joslin, E. P., *The treatment of diabetes mellitus*, Philadelphia and New York, 3rd edition, 1923.

² The writers wish to thank Miss Marguerite Fletcher of The Roosevelt Hospital for the cultures and media used in this work.

Wu method,³ and the filtrates analyzed by the Shaffer and Hartmann sugar method.⁴ The results of one series of experiments are given in Table I. They represent the averages of duplicate determinations.

In Table I, which gives at three different incubation periods, the effects of a very large variation in the insulin concentration, and for constant insulin concentration a time variation of 4, 7, and 24 hours at eight concentrations, no significant influence of iletin on the *Bacillus coli* fermentation is seen. The possible action of the iletin was studied similarly under various other conditions such as (a) starting with very heavy and very light *Bacillus coli* cultures, (b) initially using as much as 100 units of iletin per cc. of mixture, (c) adding iletin every few hours, and

TABLE I.
The Fermentation of Glucose by B. coli in the Presence of Insulin.

Experiment No.	Units of iletin per 102.5 cc. mixtures.	Amounts of glucose per 102.5 cc. mixtures.			
		After ½ hr.	After 4 hrs.	After 7 hrs.	After 24 hrs.
		gm.	gm.	gm.	gm.
1	0	1.015	0.880	0.569	0.130
2	0.5		0.872	0.564	0.084
3	1.0		0.830	0.554	0.113
4	5.0		0.840	0.534	0.113
5	10.0		0.880	0.534	0.084
6	15.0		0.835	0.554	0.084
7	25.0		0.842	0.573	0.113
8	50.0		0.825	0.554	0.130

(d) using other periods of time. The same lack of influence by iletin was observed so that these experiments will not be reported in detail. It may also be stated that in a number of experiments the Benedict and Osterberg method⁵ was used in addition to the Shaffer-Hartmann method to follow the disappearance of the glucose and in one series the carbon dioxide production was also studied. In no case was any change, caused by the iletin, observed in the fermentation.

CONCLUSION.

The fermentation of glucose by *Bacillus coli* was not influenced by insulin.

³ Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

⁴ Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920-21, xlv, 349.

⁵ Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1921, xlviii, 51.

OBSERVATIONS ON THE ORIGIN OF URINARY AMMONIA.

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(Received for publication, May 1, 1924.)

INTRODUCTION.

For many years the source of urinary ammonia has constituted a perplexing problem for physiological chemists. In 1921 Nash and Benedict¹ advanced the somewhat startling hypothesis that the kidneys themselves form the ammonia which they excrete. This idea was based chiefly on the observations made by these authors that the NH_3 content of blood taken from the renal vein of the dog is greater than that of arterial blood or of venous blood from other sources in the same animal. Furthermore, they suggested that it is quite improbable that the kidney should be able to eliminate from the arterial blood, with its low NH_3 content, the large amounts of NH_3 found in the urine. This new view-point seemed of sufficient importance to justify confirmation and further investigation.

It occurred to us that significant evidence for this theory of NH_3 formation might be derived from experiments similar to those of Nash and Benedict, but on animals which, unlike the dog, have an extremely limited capacity to excrete NH_3 by the kidneys. The rabbit excretes practically no NH_3 , and is therefore available for such a comparative study.

Procedure.

Blood NH_3 determinations were made by the method of Nash and Benedict, all the precautions suggested by them being carefully observed. A preliminary study of the method showed it to

¹Nash, T. P., Jr., and Benedict, S. R., *J. Biol. Chem.*, 1921, xlviii, 463.

be sufficiently accurate for physiological experiments of the type presented in this paper. The determinations were almost invariably made in duplicate and were always made immediately after withdrawal of the blood from the animal.

In the operative experiments the animals were anesthetized with ether and blood samples were taken as promptly as possible and with practically no manipulation of the kidney. In taking blood from the renal vein, the needle was pointed toward the kidney and the vena cava was compressed below by the finger. When blood was taken from the vena cava, back-flow from the kidneys was obviated by pressure on the cava. Urine specimens for NH_3 and pH determinations were removed from the bladder during the course of the operation.

EXPERIMENTAL.

1. *Blood Ammonia Studies in the Dog*.—Four dogs were studied and from Table I it may be seen that the NH_3 content of the renal vein is much higher than that of the arterial blood. Furthermore, it is apparent that while the NH_3 content of the vena cava and splenic veins was somewhat higher than that of arterial blood, it was very much less than that of the renal veins. Only a limited number of experiments were performed on the dog in view of the fact that this part of the work was done only to confirm the findings of Nash and Benedict.

2. *Ammonia Excretion in the Rabbit*.—Three rabbits were fasted for 3 days. On the first 2 days they received 300 cc. of water by stomach tube, given in 100 cc. amounts. On the 3rd day they received 300 cc. of 0.1 N HCl or H_2SO_4 in place of water, by stomach tube. 24 hour collections of urine were made on the 3rd day. Table II shows that starvation and even high acid feeding result in the excretion of only traces of ammonia.

3. *Blood Ammonia Studies in the Rabbit*.—Table III shows that there is only a very slight excess of NH_3 in the renal vein as compared with arterial blood and that the NH_3 excreted in the urine of the rabbits averaged about one-sixtieth of that eliminated by the dogs. There appears to be a greater fluctuation in the absolute value for the NH_3 content of arterial blood in the rabbit than in the dog, but the average is approximately the same. The cause for this fluctuation will be discussed in another paper.

TABLE I.
Study of NH_3 Content of Blood from Various Sources in the Dog.

Dog No.	Source of blood.	$\text{NH}_3\text{-N}$ per 100 cc. blood.	Urine NH_3 as cc. 0.02 N HCl per cc. urine.	Urine pH.	Remarks.
		mg.	cc.		
1	Femoral artery.	0.05	1.49		
	Renal vein.	0.16			
2	Femoral artery.	0.05	4.93	5.8	Blood taken from vena cava 15 min. after other samples were removed.
	Renal vein.	0.13			
	Vena cava.	0.11			
3	Femoral artery.	0.03	3.23	6.8	
	Left renal vein.	0.30			
	Right " "	0.30			
	Vena cava.	0.05			
4	Femoral artery.	0.01	1.45	6.6	
	Right renal vein.	0.10			
	Left " "	0.09			
	Splenic vein.	0.04			
	Vena cava.	0.04			

Average NH_3 content of femoral artery = 0.04 mg. $\text{NH}_3\text{-N}$ per 100 cc.
 " " " " vena cava = 0.07 " " " 100 "
 " " " " renal vein = 0.17 " " " 100 "
 " " " " 1 cc. urine = 2.78 cc. 0.02 N HCl.

TABLE II.
Demonstration of Low NH_3 Excretion in Fasting Rabbits after Feeding 300 Cc. of 0.1 N HCl or H_2SO_4 .

Rabbit No.	24 hr. volume of urine.	Total NH_3 as 0.02 N HCl.	NH_3 as 0.02 N HCl per cc. urine.
	cc.	cc.	cc.
A	340	6.80	0.02
B	193	5.00	0.03
C	445	8.90	0.02

TABLE III.

Comparison of NH_3 Content of Blood from the Aorta and Renal Vein of the Rabbit.

Rabbit No.	$\text{NH}_3\text{-N}$ per 100 cc. blood.		NH_3 as 0.02 N HCl in 1 cc. urine.	Urine pH.	Remarks.
	Aorta.	Renal vein.			
	mg.	mg.	cc.		
1	0.05	0.08		5.9	Arterial determination run only 4 min.
2	0.05	0.08	0.04	7.0	
5	0.06	0.04			No urine in bladder.
7	0.06	0.09	0.05	8.0	
8	0.02	0.06	0.02	7.2	
9	0.11	0.09	0.03	7.2	
10	0.05	0.04	0.04	6.8	
11	0.04	0.03	0.09	7.2	
16	0.09	0.11	0.01	7.1	
17	0.04	0.08	0.06	4.9	
19	0.01	0.03	0.05	6.3	
Average....	0.052	0.069	0.04		

DISCUSSION.

From the data presented, it is quite evident that in animals which excrete but traces of ammonia by the kidney, the NH_3 content of blood from the renal vein and aorta is almost identical, whereas, in animals excreting large amounts of NH_3 , there is a striking difference. These findings, therefore, lend support to the conception that NH_3 is formed by the kidney as has been suggested by Nash and Benedict.

The fact that the NH_3 content of the vena cava and splenic vein is slightly higher than that of arterial blood suggests the possibility that traces of NH_3 are liberated in many tissues as a result of metabolic activity. This difference is about the same as that seen in the renal vein and artery of the rabbit, which is excreting practically no NH_3 at all.

CONCLUSIONS.

1. The ammonia content of the renal vein of the dog is many times greater than that of arterial blood. This is a confirmation of the findings of Nash and Benedict.

2. The ammonia content of the blood from the renal vein of the rabbit (which excretes only traces of NH_3 in the urine) is practically the same as that of blood from the aorta.

3. Venous blood from sources other than the renal veins has a slightly higher NH_3 content than arterial blood. This is possibly a result of tissue metabolic activity.

4. The results of our experiments lend definite support to the theory that urinary NH_3 is formed by the kidney.

We are indebted to Miss E. G. Nichols and Mr. S. Steinberger for technical assistance in this work.



ANIMAL CALORIMETRY.

TWENTY-FIFTH PAPER.

THE RELATIVE SPECIFIC DYNAMIC ACTION OF VARIOUS PROTEINS.*

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(Received for publication, June 4, 1924.)

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I. INTRODUCTION.

As long ago as 1852 Bidder and Schmidt observed that giving large quantities of meat to a starved animal resulted in a marked

* Brief abstracts of this work were presented by Rapport before the National Academy of Sciences at Washington in April, 1923, and by Lusk before the International Physiological Congress at Edinburgh in July, 1923.

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rise in the respiratory gaseous exchange. The constancy of this increase in metabolism has since been amply confirmed, and to it Rubner has given the name of the *specific dynamic action* of protein. This phenomenon is not due to the combustion of the protein itself, with the subsequent evolution of heat, but, as was experimentally proved by Lusk, is due to a stimulation of the metabolism of the body cells. The investigations of Lusk (1) have further shown the special significance of different amino acids contained within the protein molecule, as regards their power to increase the heat production in the dog. On the basis of these researches Lusk came to the conclusion that the specific dynamic action of protein was due chiefly to the amino acids, glycine and alanine.

At Professor Lusk's suggestion, and in part with his aid, the effects of the ingestion of various proteins were investigated with a view to determine what relation their amino acid content bore to their influence upon heat production. The study of this problem forms the subject of this paper.

The protein materials which were considered were six in number—beef, gelatin, casein, gliadin, codfish, and chicken—and were of widely varying amino acid content. Examples of their variation in those amino acids that are of peculiar significance in this connection, namely glycine and alanine, are as follows:

Beef (2) contains approximately 4 per cent glycine and 8 per cent alanine.

Gelatin (3) contains approximately 26 per cent glycine and 9 per cent alanine.

Casein (4) contains approximately no glycine and 1.5 per cent alanine.

Gliadin (4) contains approximately no glycine and 2.0 per cent alanine.

On a *priori* grounds, therefore, it appeared that, of these proteins, gelatin should exert the greatest specific dynamic action, and casein and gliadin the least, with beef intermediate between them.

II. EXPERIMENTAL PROCEDURE.

All the experiments of this series were carried out on a single dog (Dog XIX), which had been the subject of many previous experiments, and was accustomed to lie quietly in the calorimeter over a period of many hours. Movements of the animal within the calorimeter were recorded by a device that has been described

by Williams, Riche, and Lusk (5); and when the activity of the animal was great enough to affect the metabolism appreciably the experiments were discarded. A maintenance diet, long used in this laboratory (6) for dogs of similar size, consisted of 100 gm. of lean beef heart, 100 gm. of biscuit meal, 20 gm. of lard, and 10 gm. of bone ash (5.25 gm. of nitrogen and 688 calories), and was given each afternoon at about 5 o'clock. The basal metabolism was determined approximately 18 hours after this meal. On those days when the effect of food was to be tested the food was administered about 17 hours after the ingestion of the maintenance diet and the experimental record was begun an hour later. The temperature of the calorimeter was maintained in the neighborhood of 25-26°C.

III. METHODS OF CALCULATION.

Certain possible sources of error confronted us during the course of these experiments if we were to use the ordinary methods of calculation. These were due chiefly to difficulties in the estimation of the protein metabolism from the urinary nitrogen. The excretion of nitrogen is at best an imperfect criterion of the rate of metabolism of the ingested protein from which that nitrogen is derived—at least during the early hours of digestion. Reilly, Nolan, and Lusk (7) have shown that in a phlorhizinized dog the actual metabolism of protein during the first hours after its ingestion, as measured by the appearance of urinary sugar formed from it, was relatively larger in amount than was indicated by the urinary nitrogen originating from the same protein; also, Hohlweg and Meyer (8) found that there was an accumulation of urea in the blood following meat ingestion, with a consequent delay in the nitrogen excretion.

It was convenient for the purposes of our research, as will be brought out later, to calculate the total protein metabolism upon the assumption that the urinary nitrogen per hour was the average of the output during the experimental period. The dog was catheterized before entering and after leaving the calorimeter. Williams, Riche, and Lusk (5) showed that the error introduced as a result of using this method of calculation was inappreciable, a conclusion which we were able to confirm. Thus, Experiment 98, when calculated upon the average nitrogen output of

the experimental period of 5 hours, showed total calories of 22.34; when calculated upon the nitrogen output of the 2nd hour, 22.59; and when calculated upon the nitrogen output of the 5th hour, 22.32. This last probably represents most accurately the actual hourly metabolism of protein; and the nitrogen excretion of this

TABLE I.
A Method of Determining Factors for Gelatin.

	C	H	O	N
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Excretion products of meat in urine and feces (9).....	10.88	2.88	14.99	16.65
Composition of gelatin.....	42.86	5.71	20.94	15.49*
Excretion products of gelatin in urine and feces†.....	10.36	2.67	13.95	15.49
	32.50	3.04	6.99	
Deduct intramolecular water.....		0.87	6.99	
Oxidized to CO ₂ and H ₂ O.....	32.50	2.17		
8 × 2.17 = 17.36 gm. O ₂ required to produce H ₂ O.				
32/12 × 32.50 = 86.67 " " " " " CO ₂ .				
104.03 " " in respiratory exchange for each 100 gm. gelatin.				
86.67 + 32.50 = 119.17 gm. CO ₂ in respiratory exchange for each 100 gm. gelatin.				
R. Q.	= 0.833			
100 gm. gelatin	= 341.80 calories.‡			
1 liter respiratory O ₂	= 4.694 "			
1 gm. N in urine	= 6.46 gm. gelatin = 22.08 calories;			
and in respiration	{ 3.92 liters (7.70 gm.) CO ₂ .			
	{ 4.70 " (6.72 ") O ₂ .			

* N actually found in commercial gelatin used. Percentages of other elements calculated (10) as though the material had been pure gelatin.

† Assuming the excretion products in relation to nitrogen to have been the same as those of meat protein.

‡ Calculated from the figures of Krummacher (11).

hour does not, unless excessive amounts of protein are given differ sufficiently from the average excretion during the experimental period to affect appreciably the calculation of the total metabolism.

Another complication arose when different kinds of proteins were ingested, for 1 gm. of nitrogen in the urine then represents

slightly different heat values, as well as different respiratory CO_2 and O_2 values.

Table I gives a method for arriving at the factors for such a protein as gelatin, assuming that the excretion products in the urine and feces are identical with those of meat. This calculation is the most nearly accurate that can be made with the data at our disposal. The errors involved do not seriously interfere with the computations. Thus, in one experiment in which gelatin was given, the calories found, when calculated upon these factors for gelatin, were 20.86; when calculated upon the factors for meat, they were 21.19. The variation in this case is only about 1.5 per cent. In another experiment in which casein was ingested the calories found when the calculation was based on the factors for casein (9) were 21.44; when based on the factors for meat they were 21.41. In view, therefore, of the small error involved, the use of special factors was avoided in the cases when proteins other than meat were given, and the experiments were calculated upon the assumption that all the protein metabolism had been in the form of meat protein.

IV. THE EXPERIMENTS.

A. The Basal Metabolism.

The constancy of the basal metabolism was very striking (Table II). These determinations cover a space of 15 months. The average heat production was 16.52 calories, and from this mean the maximum variation was +0.47 calories and -0.49 calories, ± 2.9 per cent, or hardly greater than the variation in the determinations of oxygen during alcohol checks. In eight of the experiments the maximum deviation was +0.4 per cent and -0.7 per cent.

These results may possibly give pause to authors such as Lefèvre (12), for example, and others in our own country and induce them to reexamine the evidence which has led them to suspect the inconstancy of the basal metabolism and to proclaim its inadequacy as a standard of measurement.

Lusk (13) has called attention to the fact that if an animal that has been accustomed to a vigorous outdoor life is immured in a cage, the basal metabolism will tend gradually to drop until,

after several weeks, it will reach a certain level, at which it will thereafter remain, given constant conditions of life and an unvarying body weight. This phenomenon we observed in the present instance, there being a gradual decline in the dog's metabolism for about 3 or 4 weeks after she was brought in from the country

TABLE II.

*The Basal Metabolism of Dog XIX (Weight 11.5 Kilos) during 1923-24.**

Experiment No.	Date.	R.Q.	Calories per hr.	
			Indirect.	Direct.
1923				
96	Jan. 30	0.83	16.58	17.08
102	Feb. 14	0.82	16.42	15.98
107	" 21	0.80	16.44	16.34
110	Mar. 2	0.81	16.13	17.46
115	" 13	0.79	16.76	18.01
122	" 24	0.80	16.46	16.60
129	Apr. 4	0.88	16.52	17.02
146	May 9	0.83	16.74	14.26
Average for 1923.....		0.82	16.51	16.72
1924				
167	Dec. 5	0.79	16.39	15.92
175	Mar. 6	0.81	16.43	15.71
181	" 27	0.88	16.57	16.01
185	Apr. 3	0.84	16.99	14.40
190	" 8	0.89	16.78	16.52
196	" 24	0.89	16.03	
Average for 1923-24.....		0.85	16.53	15.71
" " 15 mos.....		0.83	16.52	16.21

* The specific dynamic action of the substances tested in this research has been calculated on a basal value of 16.48 calories (the average of Experiments 107, 110, 115, 122, and 129). The inclusion of the other basal determinations does not materially change this average.

(where she spent 6 months during the summer of 1922) from a basal level of 20.5 calories to the one of 16.5, as charted in Table II, from which she did not deviate throughout the winter. During the summer of 1923 the dog again lived in the country, and again cage life in the laboratory produced the same change as in

the preceding year. Thus, the basal metabolism, which on October 31, 1923, was 20.6 calories, gradually declined until December 5, when it reached 16.4 calories, at which level it subsequently remained constant. The phenomenon of "cage life" unquestionably has its counterpart in the human family also.

B. The Effect of Ingesting Various Proteins.

Rubner (14) found that the specific dynamic action of gelatin was almost the same as that of meat, and Falta, Grote, and Staehelin (15) made a similar observation with casein. We have systematically investigated proteins of both animal and vegetable origin, and the results we obtained are summarized in Chart I.

In this series of experiments we gave in each case an amount of protein material that contained 6 gm. of nitrogen. In the case of the dry proteins sufficient water was added to make the water content of the ingested substance approximately equal to that of 200 gm. of beef; 2 gm. (0.156 gm. of nitrogen) of Liebig's extract of beef were also added to these proteins to supply flavor. Lusk (1) has shown that, when taken in this amount, Liebig's extract has a negligible influence upon the metabolism and does not cause the nitrogen output to increase more than 0.04 gm. per hour, a factor that has been neglected in our calculations.

1. *Beef*.—200 gm. of beef heart, cut free from fat and connective tissue, were given, and the determinations of the metabolism were begun 1 hour later. The heat production in three experiments rose to 22.3, 21.5, and 22.3 calories per hour, increases of 35.6, 30.3, and 35.6 per cent above the basal metabolism. The average increase was 33.8 per cent (Table III).

2. *Casein*.—In contradistinction to beef, casein contains no glycine whatever and only 1.5 per cent alanine. Yet in two experiments after giving this substance, the heat production rose to 21.7 and 21.4 calories, an average increase of 30.7 per cent over the basal metabolism (Table IV).

3. *Gliadin*.—Gliadin was a rather difficult substance to give. The dry powder, when mixed with water, formed a gummy, stringy mass which the dog would not eat and which could not be introduced by stomach tube. We found, however, by adding

R.Q.	0.80	0.86	0.80	0.81	0.82	0.81	0.83
— 1648.	21.30	21.54	21.59	22.05	22.21	22.30	
---- 16.86	21.00	21.42	21.65	22.35	22.15	21.20	
% INCREASE OVER BASAL.	29.22	30.67	31.01	33.80	34.77	35.32	
CAL. 25 —							
	— HEAT CALCULATED.						
	---- " FOUND.						

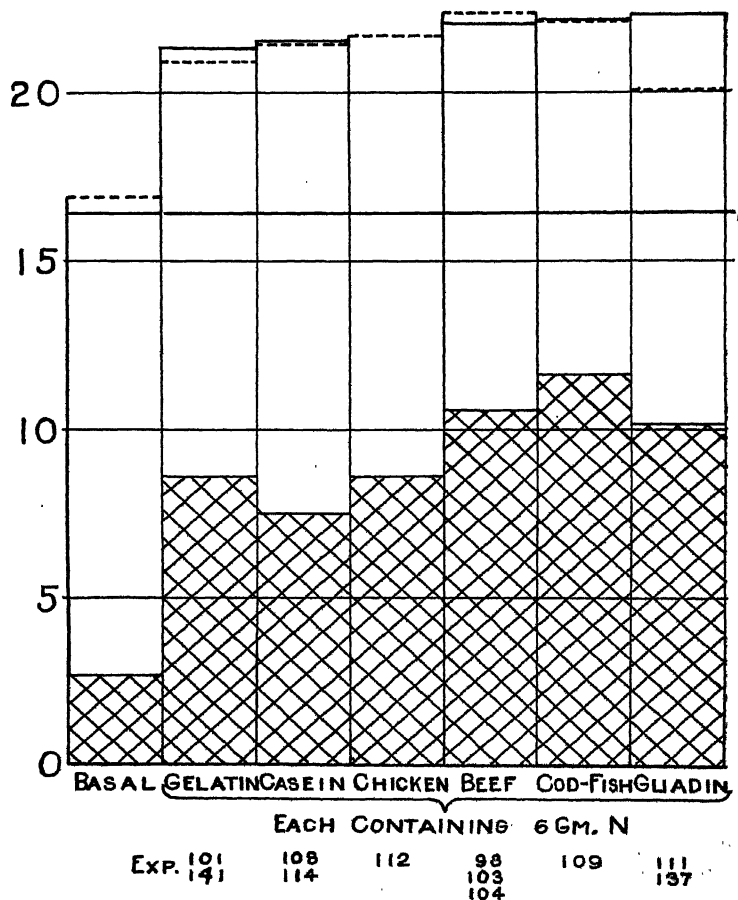


CHART I. The rise in heat production after giving beef, gelatin, casein, gliadin, chicken, and codfish in amounts containing 6 gm. of nitrogen.

— Calories by indirect; --- calories by direct calorimetry. Calories of protein metabolism in crisscrossed portions.

100 cc. of 0.5 per cent HCl to the 37.5 gm. of gliadin which composed the test diet, and whipping this with a glass rod, that a mixture was created which, while still somewhat sticky, was frothy and light, and not unlike a sort of thin batter. This concoction, when flavored with Liebig's extract, the dog lapped with avidity.

We were here dealing with a protein which, like casein, has no content of glycine, has only 2.5 per cent of alanine, and, moreover,

TABLE III.
Beef, 200 Gm.

(Average per hour of 4 hour period.)

Experiment No.	Date.	R.Q.	CO ₂	O ₂	Urinary N.	Calories.		Increase over basal.
						Indirect.	Direct.	
	1923		gm.	gm.	gm.			per cent
98*	Feb. 2	0.81	7.64	6.87	0.409	22.34	22.41	35.6
103*	" 15	0.84	7.48	6.47	0.252	21.47	22.58	30.3
104†	" 17	0.83	7.75	6.82	0.388	22.34	22.24	35.6
Average.....			7.62	6.72		22.05	22.40	33.8

* 2nd and 3rd hours after food. Animal restless thereafter.

† 2nd, 3rd, and 4th hours after food. Animal restless thereafter.

TABLE IV.
Casein, 48.67 Gm.

(Average per hour of 4 hour period.)

Experiment No.	Date.	R.Q.	CO ₂	O ₂	Urinary N.	Calories.		Increase over basal
						Indirect.	Direct.	
	1923		gm.	gm.	gm.			per cent
108	Feb. 27	0.80	7.26	6.58	0.285	21.66	21.13	31.4
114	Mar. 8	0.81	7.23	6.51	0.268	21.41	21.71	29.9
Average.....			7.25	6.55	0.277	21.54	21.42	30.7

contains 44 per cent of glutamic acid, which Lusk (1) found to have practically no influence upon the heat production. Nevertheless, the metabolism, following the ingestion of gliadin, showed an average increase of 35.3 per cent over the basal, an increase that was the same as in two of the three experiments after giving beef (Table V).

a. *The Effect of HCl Alone and When Added to Casein.*—The necessary addition of HCl to the gliadin mixture in order to make it more edible introduced a foreign element that required investigation. Atkinson and Lusk (16) found that 200 cc. of 0.4 per

TABLE V.
Gliadin, 37.5 Gm.

(Average per hour of 4 hour period.)

Experiment No.	Date.	R.Q.	CO ₂	O ₂	Urinary N.	Calories.		Increase over basal.
						Indirect.	Direct.	
	1923		gm.	gm.	gm.			per cent
111	Mar. 3	0.83	7.78	6.84	0.395	22.36	21.60	35.7
137	Apr. 17	0.84	7.80	6.77	0.375	22.24	20.85	35.0
Average.....			7.79	6.81		22.30	21.22	35.3

TABLE VI.
The Effect of 0.5 Gm. of Hydrochloric Acid.

(Average per hour of 4 hour period.)

Food.	Experiment No.	Date.	R.Q.	CO ₂	O ₂	Urinary N.	Calories.		Increase over basal.
							Indirect.	Direct.	
		1923		gm.	gm.	gm.			per cent
Basal.....	Average.		0.80	5.57	4.92	0.103	16.48	16.96	
HCl, 0.5 gm.....	113*	Mar. 7	0.79	5.75	5.31	0.107	17.61	17.89	6.9
Casein, 43.67 gm.	Average of Nos. 108 and 114.		0.80	7.25	6.55	0.277	21.54	21.42	30.7
" 43.67 " and HCl, 0.5 gm.....	116†	Mar. 14	0.80	7.07	6.46	0.285	21.13	20.89	28.2

* 2nd hour after HCl was administered. Animal restless thereafter.

† 3rd, 4th, and 5th hours after food.

cent HCl increased the hourly metabolism in a dog from 20 to 21.25 calories; and Lusk (17) later gave to the same dog that was used in the present experiments 300 cc. of 0.6 per cent HCl with a consequent rise in the heat production of 1.55 calories per hour. Upon ingesting 100 cc. of 0.5 per cent HCl we obtained an increase

of 1.31 calories or 6.9 per cent above the basal level (Table VI). It appeared that this might have to be reckoned with in the calculation of the gliadin results.

We observed that the dog, while not active during the experiment with hydrochloric acid, appeared to be wakeful and alert. It seemed possible that such influences as these would tend to raise the metabolism and might be due to an irritating effect of the acid upon the stomach, which might be eliminated by the neutralizing effect of the acid with a protein. Accordingly, the same quantity of 0.5 per cent HCl (100 cc.) was added to casein, which had already been studied and found to increase the metabolism over 29 per cent. The mixture was similar in physical appearance to that when HCl was added to gliadin, and the in-

TABLE VII.

Codfish, 218.2 Gm., and Chicken, 157.1 Gm.

(Average per hour of 4 hour period.)

Food.	Experiment No.	Date.	R.Q.	CO ₂	O ₂	Urinary N.	Calories.		Increase over basal.
							Indirect.	Direct.	
		1923		gm.	gm.	gm.			per cent
Codfish.....	109	Mar. 1	0.82	7.65	6.84	0.439	22.21	22.15	34.8
Chicken.....	112	" 6	0.81	7.37	6.58	0.327	21.59	21.64	31.0

crease of heat production when it was given was 28 per cent (Table VI), an increase that was practically the same as when casein was given alone. Therefore it seems reasonable to suppose that the quantity of HCl that was added to the gliadin had no appreciable influence upon the specific dynamic action of the latter, particularly in view of the fact that during the experiments determining the effect of this protein the dog was extremely quiet.

4. *Codfish and Chicken.*—According to Osborne and Jones (18), fish contains no glycine and a questionable trace of alanine, while chicken contains only 0.68 per cent glycine and 2.28 per cent alanine. Nevertheless, the specific dynamic action of both codfish and chicken proved to be substantially the same as that of beef. Thus, when 218.2 gm. of codfish were given, the metabolism rose to a level of 22.21 calories per hour, an increase

of 34.8 per cent over the basal; and when 157.1 gm. of chicken were ingested, the heat production was raised to 21.59 calories per hour, an increase of 31 per cent (Table VII). Both foods contained 6 gm. of nitrogen.

5. *Gelatin*.—Gelatin contains approximately 26 per cent of glycine; nevertheless its behavior was not markedly different from that of the other proteins studied. In fact, it showed a slightly lower specific dynamic action than did any of the others, the average increase in two experiments being 29 per cent (Table VIII).

a. *The Effect of Ingesting Cystine with Gelatin*.—Gelatin is an incomplete protein, lacking, among other amino acids, cystine. The recent observations of Hopkins (19) have shown that a com-

TABLE VIII.
Gelatin, 38.74 Gm.

(Average per hour of 4 hour period.)

Experiment No.	Date.	R.Q.	CO ₂	O ₂	Urinary N.	Calories.		Increase over basal.
						Indirect.	Direct.	
	1923		gm.	gm.	gm.			per cent
101	Feb. 9	0.85	7.55	6.43	0.371	21.19	21.68	28.6
141*	Apr. 30	0.87	7.72	6.48	0.381	21.41	20.30	29.9
Average.....			7.64	6.51		21.30	20.99	29.3

*2nd and 3rd hours after food. During the 4th and 5th hours the dog was active.

bination of glutamic acid with either cystine or its reduced form, cysteine, exists in wide distribution though in low concentration in animal tissues. This substance—glutathione—can act as a reducing agent in its reduced form, or as a hydrogen acceptor and catalyzer of oxidation in its oxidized form.

In view of the fact that the formation of this substance could not take place at the expense of the gelatin molecule, it seemed possible that the specific dynamic action of the gelatin might be increased by adding 1 gm. of cystine to the quantity of gelatin that was ordinarily ingested. If glutathione were subsequently formed, it might exert a catalytic function upon the oxidations within the body cells, and thus increase the heat production above

that which resulted from the ingestion of gelatin alone. There was also the possibility that glutathione might hasten the breakdown of the amino acids of the gelatin itself, and thus indirectly increase the specific dynamic effect by flooding the tissue cells

TABLE IX.

Comparison of Gelatin Given Alone and with Cystine.

(Average per hour of 4 hour period.)

Food.	Experiment No.	Date.	R.Q.	CO ₂	O ₂	Urinary N.	Calories.		Increase over basal.
							Indirect.	Direct.	
		1923		gm.	gm.	gm.			per cent
Gelatin.....	Average of Nos. 101 and 141.*		0.86	7.64	6.46	0.371	21.30	20.99	29.2
Gelatin and cystine.....	143	May 2	0.86	7.63	6.47	0.354	21.38	21.82	20.7

* See Table VIII.

TABLE X.

The Nitrogen Elimination.

No. of experiments.	Diet.	Urine collection in hrs. after food.	Nitrogen per hr.
	gm.		gm.
18	Basal.		0.10
1	HCl, 0.5	3½	0.11
2	Casein, 43.67	5½	0.28
1	Chicken, 157.1	5½	0.33
2	Gelatin, 38.74	5½	0.37
3	Beef, 200	5½	0.39
2	Gliadin, 37.5	5½	0.39
1	Codfish, 218.2	5	0.44

with the intermediate products of gelatin digestion, which would in turn stimulate the activity of the cells.

The results, however, proved to be negative (Table IX). Gelatin alone increased the heat production 29 per cent and, when ingested with 1 gm. of cystine,¹ the resultant rise in the heat production was 30 per cent or virtually the same.

¹ Kindly given to us by Dr. S. R. Benedict.

6. *The Nitrogen Elimination.*—Table X gives the figures for nitrogen elimination. It will be seen that the elimination varies considerably for different proteins in spite of the constancy of the increase in metabolism when they are administered.

C. Alcohol Checks.

The soundness of the data obtained in these experiments is established by the alcohol checks carried out during the period of the experiments (Table XI).

TABLE XI.
Alcohol Checks.

Experiment No.	Date.	R.Q.	Calories per hr.		Difference.
			Indirect.*	Direct.	
	1923				cals.
168	Jan. 4	0.663	25.15	23.45	-1.70
169	" 5	0.672	27.16	27.94	+0.78
170	" 20	0.678	25.10	25.42	+0.32
171	" 25	0.662	30.54	31.03	+0.49
172	Mar. 9	0.668	25.75	26.08	+0.33
173	May 3	0.659	24.90	25.42	+0.52
174	" 11	0.670	23.42	23.35	-0.07
175	" 22	0.666	21.65	22.37	+0.72
Average.....		0.667	25.46	25.63	+0.17

* Calculated upon the CO₂.

Maximal O₂ error = -1.86 per cent (Experiment 170).

" " " = +1.15 " " (" 173).

V. CONCLUSIONS.

1. Six proteins of both animal and vegetable origin, namely beef, casein, gliadin, codfish, chicken, and gelatin, when given to a dog in portions each of which contained 6 gm. of nitrogen, showed approximately the same specific dynamic action. These proteins are of widely differing amino acid content.

2. The addition of cystine to gelatin did not increase the specific dynamic action of the gelatin. If glutathione was formed under these circumstances its lack of stimulating effect upon the metabolism may have been due to the fact that it was already present in optimum concentration in the tissues (Hopkins.)

3. 0.5 gm. of hydrochloric acid in 100 cc. of water, when given alone, increased the metabolism slightly, but it was observed that, although the dog was not active, she was alert—due, possibly, to the irritating effect of the acid—which is held to account for the increase. When mixed with casein this quantity of hydrochloric acid did not add to the specific dynamic action of the casein.

4. Attention is called to the absolute constancy of the basal metabolism over a period of 15 months.

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ANIMAL CALORIMETRY.

TWENTY-SIXTH PAPER.

THE INTERRELATIONS BETWEEN CERTAIN AMINO ACIDS AND PROTEINS WITH REFERENCE TO THEIR SPECIFIC DYNAMIC ACTION.

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I. INTRODUCTION.

In the preceding paper of this series one of us has demonstrated that the specific dynamic action of proteins of varied amino acid content is not proportional to their content of glycine and alanine, both of which amino acids are powerful stimulants to a higher heat production in the animal body. The present paper is an attempt to elucidate this anomaly. It might be that the similarity of widely differing proteins is due to a constant and equal synthetic production from them of the amino acids, glycine and alanine. Csonka, however, in a paper to follow this one, has shown that when a pig is continuously given benzoic acid the quantity of glycine synthesized from casein is far less than the amount which is produced and eliminated after the administration of gelatin. In the present work an attempt has been made to investigate whether preformed glycine in gelatin reacts the same way as ingested glycine. For example, 10 gm. of glycine induce about one-half the increase in heat production of that after giving 20 gm. If the action of preformed glycine in gelatin were the same as crystalline glycine, then when the two materials were administered in portions each of which was the equivalent of 10 gm. of glycine, there should be a much larger increase in heat production than if either were given alone. To verify the results obtained gelatin was also given *per os* at the same time that glycine was administered parenterally, either subcutaneously or intravenously. For comparison, alanine and casein were also used as foods in the course of the experiments. The outcome was such as to lead to the investigation of the behavior of the body when two half portions of meat and casein were given together, and this result was compared with the levels of metabolism reached after administering one portion of each food material. And this was followed by an investigation into the reaction of the body when glycine and asparagine were given together. The outcome of the investigation shows that the physiological behavior of the isolated amino acids is different from that of the polypeptides formed in digestive proteolysis.

The experimental procedure did not differ markedly from that adopted in previous papers of this series. The maintenance diet was varied in so far as it contained 20 gm. of butter instead of

lard, in addition to the customary 100 gm. of biscuit meal, 100 gm. of beef heart, and 10 gm. of bone ash. For some months 1 gm. of yeast vitamin (Harris) was added to the daily diet, but this was later discontinued. The administration of the yeast had no apparent effect either upon the general condition of the dog or upon the basal metabolism. Experiments were begun about 18 hours after the administration of the maintenance diet, and the precautions hitherto described in reports from this laboratory were taken to exclude the influence of muscular activity and external temperature while the animal was in the dark chamber of the calorimeter.

II. METHODS OF CALCULATION.

Certain of the difficulties inherent in the calculation of the protein metabolism have been described in the preceding paper of this series (1) when a single protein was administered. Even if it were feasible to use the factors appertaining to a particular protein when it alone is given, it becomes still more complicated when two or more proteins are given together, for we have no means of determining what percentage of each is being metabolized in a given time. Hence, the experiments, for the most part, were calculated upon the average hourly excretion of nitrogen as though meat had been the metabolized protein. The error, as has been pointed out, is negligible.

In those experiments in which successively larger quantities of meat up to 800 gm. were administered, the calculations were based on the nitrogen excretion of the 5th hour after the intake of the diet.

Another exception was made to the general rule. The rates of metabolism of glycine and alanine have been established with a fair degree of accuracy by Csonka (2) on the basis of the rapidity of the excretion of sugar derived from these amino acids in phlorhizin diabetes. In those experiments in which glycine or alanine alone, or glycine and alanine together, were given, the calculations were based on Csonka's figures, as described by Lusk (3). Nevertheless, it is interesting to note (Table I) that here also the total metabolism remains nearly the same whatever the method of calculation, although the allocation of calories to "protein" and

"non-protein" metabolism varies considerably, and, in the case of glycine, the non-protein respiratory quotient also changes.

In all the accompanying charts the portions crisscrossed represent calories of protein or amino acid metabolism.

TABLE I.

Comparison of Experiments Calculated upon Factors for Meat and for Amino Acids.

	R. Q.	Non-protein* R. Q.	Calories.		
			Protein.	Non-protein.	Total.
I. Glycine.					
(Experiment 119) <i>a.</i> Calculated on meat factors.....	0.88	0.95	9.09	10.67	19.76
<i>b.</i> Calculated on glycine factors.....	0.88	0.85	6.51	13.47	19.98
II. Alanine.					
(Experiment 125) <i>a.</i> Calculated on meat factors.....	0.83	0.85	11.24	9.79	21.03
<i>b.</i> Calculated on alanine factors.....	0.83	0.83	8.43	12.87	21.30

* The phrase "protein," used for convenience, indicates the combined metabolism of protein and amino acid; and "non-protein," the carbohydrate and fat metabolism.

III. EXPERIMENTAL PART.

A. The Basal Metabolism.

In the preceding paper of this series attention has been called to the constancy of the basal metabolism in Dog XIX over a period of 15 months. This period included the present research, and the figures given in the table published in that paper are in part those of basal determinations made during the course of the experiments that we are now reporting. The average of all the basal determinations is 16.52 calories an hour; that of the determinations made during the present experiments was 16.53 calories an hour. This similarity and the lack of noteworthy deviation

in individual determinations has led us to adopt the same basal level that was used in the preceding paper as a basis for measuring percentage increases; namely, 16.48 calories.

B. Ingestion of Glycine or Alanine with either Casein or Gelatin.

While the evidence for the synthesis of alanine in the body is slight, it is known that a synthesis of glycine can occur. For example, a new-born baby, subsisting on a pure milk diet, the proteins of which contain no glycine, is capable of adding to its own store of body protein, which, of course, contains glycine. It is obvious that glycine must have been built within the baby's body. Moreover, the synthesis of glycine in the animal body has been indicated by many experiments in which benzoic acid was administered. It has been computed by Lusk (4) from the figures of various workers that as much as "35, 37 and 38 per cent of the total endogenous protein metabolism of man, goat and pig may pass through a glycine stage and be eliminated in the urine" when benzoic acid is ingested. Since the body proteins and ingested proteins, in these cases, contained only about 4 per cent of glycine, a synthetic production of glycine must have taken place.

If glycine were formed in the metabolism of casein, then if additional glycine were added to the diet, there might be a summation of effect, such as Lusk has found after doubling the ingested quantity of glycine. To test this proposition, casein, which alone had given an increase of 30.7 per cent above the basal metabolism, and 10 gm. of glycine, which alone induced an increase of 21.4 per cent, were given, together with 2 gm. of Liebig's extract and 150 cc. of water. The rise in the heat production induced by this mixture was 29.8 per cent (Table II). There was no summation of effect whatever.

One might argue from this that the ingestion of glycine with casein merely rendered glycine synthesis unnecessary and that the quantity metabolized was in both instances the same. This is not true because Csonka (see the following paper) finds a much greater elimination of hippuric acid after giving glycine with casein and benzoic acid to a pig than if casein and benzoic acid are given together.

Lusk (3) found that the ingestion of 20 gm. of glycine produced twice the specific dynamic action that the ingestion of 10 gm. of

TABLE II.
The Ingestion of Casein with Glycine and Alanine.

(Average per hour of 4 hour period.)

Food.	Experiment No.	Date.	R. Q.	CO ₂	O ₂	Urinary N.	Calories.		In-crease over basal.
							In-direct.	Direct.	
		1923		gm.	gm.	gm.			per cent
Glycine, 10 gm.....	119, 170, 176		0.87	7.15	5.99	0.310	20.01	19.29	21.42
Casein, 43.67 gm. (6 gm. N).....	Average of Nos. 108 and 114.		0.80	7.25	6.55	0.277	21.54	21.42	30.67
“ 43.67 “ and glycine, 10 gm.....	117	Mar. 16	0.84	7.50	6.52	0.377	21.39	20.34	29.79
Alanine, 16.85 gm.....	Average of Nos. 171 and 173.		0.86	7.05	6.01	0.352	19.97	20.08	21.14
Casein, 43.67 gm., and alanine, 16.85 gm.....	126	Mar. 29	0.82	7.62	6.71	0.370	21.99	22.39	33.44

Gelatin, 38.74 gm., and alanine, 16.85 gm.....	124	Mar. 27, 1923	0.85	7.66	6.57	0.453	21.46	22.04	30.2
Glycine, 10 gm., and alanine, 16.85 gm.....	130	Apr. 5, 1923	0.87	8.00	6.73	0.550	21.94	21.93	33.1
" 10 " " 16.85 "	172	Dec. 13, 1923	0.89	8.09	6.59	0.500	21.67	21.08	31.5
Average.....									32.3
Gelatin, 38.74 gm., glycine, 10 gm., and alanine, 16.85 gm.....	128†	Apr. 3, 1923	0.85	7.84	6.72	0.434	22.06	22.04	33.9
Gelatin, 10 gm.....	121*	Mar. 23, 1923	0.88	6.72	5.54	0.274	18.36	19.39	11.4
" 10 "	123†	" 26, 1923	0.85	6.60	5.64	0.227	18.57	17.39	12.7
" 10 " and glycine, 10 gm.....	120	" 22, 1923	0.88	7.70	6.36	0.435	20.84	21.91	26.5

* 2nd, 3rd, and 4th hours. Dog active during 5th hour.

† 3rd, 4th, and 5th hours after food.

‡ 2nd and 3rd hours. Dog active thereafter.

R.Q.	0.80	0.86	0.87	0.87	0.85	0.86	0.88	0.87	0.87	0.85
—	16.48	21.30	21.12	20.01	21.46	20.00	21.81	21.57	22.51	22.06
----	16.86	21.00	20.86	19.30	22.04	20.10	21.13	21.34	22.63	22.64
% INCREASE OVER BASAL	29.22	28.16	21.42	30.22	21.14	32.31	30.89	36.53	33.86	
CAL.										
25—	HEAT CALCULATED.									
	---- " FOUND.									

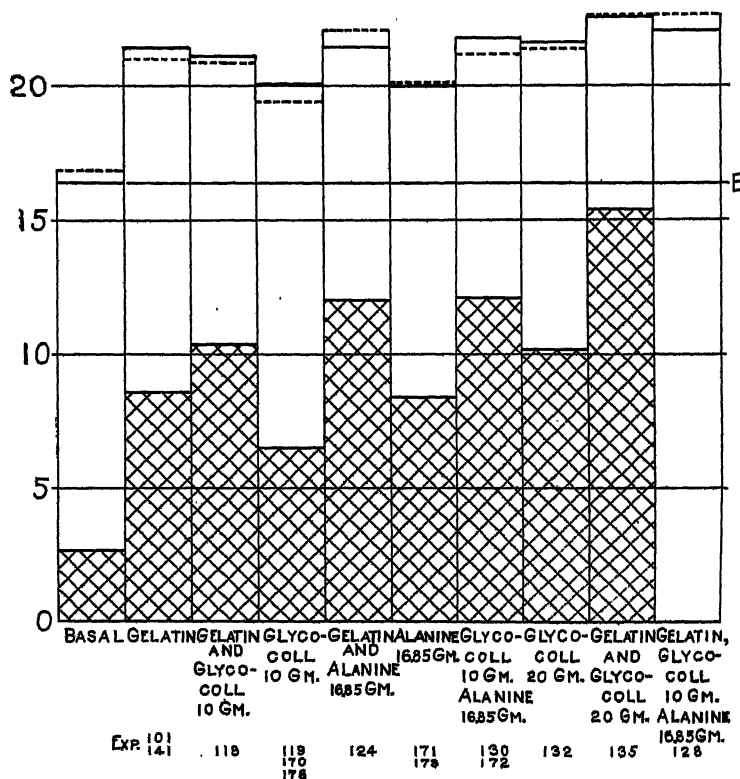


CHART II. The effect of ingesting glycine with alanine; and of ingesting glycine or alanine, or both together, with gelatin.

heat production on giving 10 gm. of glycine was 21.42 per cent and on giving 20 gm., was 30.89 per cent (Table III).

It is clear that upon doubling the amount of glycine administered there was a very considerable increase in the specific dynamic

action. When we gave the dog 10 gm. of glycine, mixed with 38.74 gm. of gelatin—which latter would have yielded, on hydrolysis, 10 gm. of glycine—the result was practically the same as when gelatin was given alone (Table III). Thus, 38.74 gm. of gelatin increased the metabolism by 29 per cent; gelatin and glycine increased it by 28 per cent.

The lack of summation when glycine was added to the protein of the diet was not due to the physical effect of incorporating the dissolved glycine into the gelatin mixture. For (Table III) when we gave 10 gm. of glycine with a small amount (10 gm.) of gelatin, the specific dynamic action of the mixture was greater than that of 10 gm. of gelatin given alone, and greater also than that after giving glycine alone. Gelatin evidently was not given in sufficient quantity to neutralize the effect of ingested glycine and a summation effect ensued. When a large amount of glycine (20 gm.) was added to 38.74 gm. of gelatin, an increase in the specific dynamic effect was likewise observed (Table III). 20 gm. of glycine had increased the metabolism 30 per cent; 38.74 gm. of gelatin had increased it 29 per cent; the mixture of the two increased it 36 per cent. Evidently, after giving 20 gm. of glycine the neutralization of its specific dynamic action did not appear to be complete. There must be something in the gelatin which is capable of neutralizing the effect of added glycine.

A critical analysis of the results reveals the following relations.

Food.	Increase in metabolism.
	<i>per cent</i>
Glycine, 10 gm.	21
" 20 "	31
Gelatin, 38.7 "	29
" 38.7 " + 10 gm. glycine.....	28
" 38.7 " + 20 " "	36.5
" 10.0 "	12
" 10.0 " + 10 " "	26.5

In view of the powerful effect of alanine upon metabolism, the result of giving it with a protein was also investigated, with findings similar to those that were obtained when glycine was employed. An amount of *L*-alanine (16.85 gm.) containing the same number of metabolizable molecules as 10 gm. of glycine

assuming 70 per cent of the ingested *l*-alanine to be metabolizable (3), when given to the dog showed an increased heat production of 21 per cent (Table I); given with casein, the increase was 33 per cent over the basal; while casein alone induced an increase of 31 per cent. When alanine was given with gelatin the rise in heat production was 30 per cent (Table II), while gelatin showed a 29 per cent increase. Again the lack of summation is evident.

The specific dynamic action of casein or gelatin, when given with glycine or alanine, may be no greater than if the protein be ingested alone.

If the quantity of gelatin is small in relation to the amount of glycine administered, a true summation occurs, indicating that the protein contains a material capable of neutralizing the specific dynamic effect of glycine.

C. Ingestion of Glycine and Alanine with Gelatin.

Gelatin (38.74 gm.) was mixed with 10 gm. of glycine and 16.85 gm. of alanine. The ingestion of this mixture produced (Table III) a specific dynamic action that was hardly greater than that of gelatin alone, or of the two amino acids administered together. Thus, gelatin increased the heat production 29 per cent; glycine and alanine ingested together increased it 32 per cent; the mixture of all three increased it 34 per cent. There was no summation of effect, for if the specific dynamic action of each of these substances—when ingested alone—had been added (gelatin, 29 per cent; glycine, 21 per cent; alanine, 21 per cent), the metabolism would have been raised 71 per cent.

D. Ingestion of Beef in Increasing Quantities.

We have seen that an amino acid and a protein, given together, mask each other's effect. We have also noted that increasing amounts of an amino acid produce successively greater specific dynamic actions. It seemed advisable to reinvestigate the effect of giving successively greater amounts of an individual protein. Rubner laid down the dictum that the specific dynamic action of a protein is proportional to the amount ingested. We gave beef in increasing quantities, beginning with 200 gm., and then in following experiments giving 400, 600, and 800 gm., respectively. The results are summarized in Table IV.

TABLE IV.
The Ingestion of Increasing Quantities of Beef.

(Average per hour of 4 hour period.)

Food.	Experiment No.	Date.	R. Q.	CO ₂	O ₂	Urinary N 5th hr. after food.	Non-protein.		Total calories.		Increase over basal.
							R. Q.	Calories.	Indirect.	Direct.	
Basal.....	Average.	1923	0.80	gm.	gm.	gm.					per cent
Beef, 200 gm.....	145*	May 8	0.81	7.42	6.61	0.470	0.84	8.94	21.40	22.02	29.8
" 400 "	144	" 4	0.82	8.05	7.16	0.735	0.91	3.26	22.75	23.63	38.1
" 600 "	147	" 10	0.81	8.90	7.96	0.986	(0.84†)	(1.23†)	24.91	24.87	51.2
" 800 "	148	" 16	0.83	9.66	8.50	1.277	(0.72†)	(7.56†)	26.29	26.84	59.5

* 2nd, 3rd, and 4th hours after food. Dog active during 5th hour.

† Deposited material.

Animal Calorimetry

	R.Q. 0.80	0.81	0.82	0.81	0.83
Non-Prot.	R.Q.	0.82	0.91	(0.84)	(0.72)
—	16.48	21.40	22.75	24.91	26.29
----	16.86	22.02	23.63	24.87	26.84

% INCREASE
OVER BASAL 29.84 36.05 51.15 59.53

CAL.

— HEAT CALCULATED
30 — " FOUND

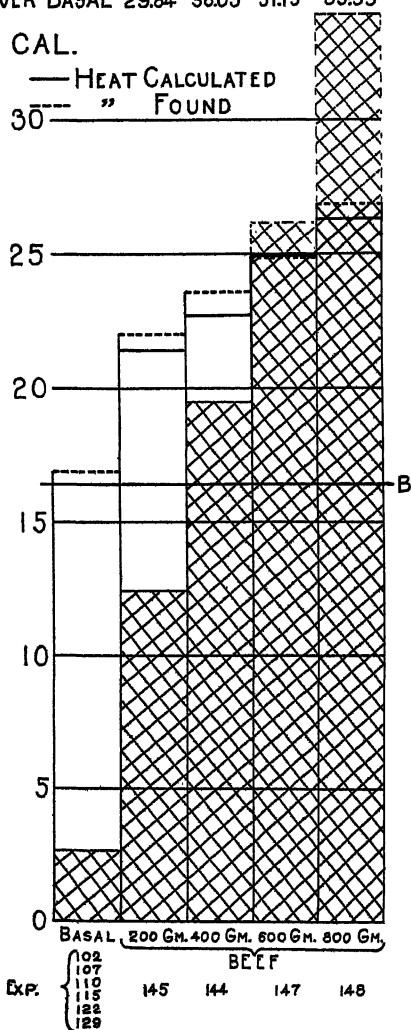


CHART III. The rise in heat production after giving 200, 400, 600, and 800 gm. of beef.

The increase in the heat production on giving 200 gm. of beef on May 8 was 30 per cent. With each additional 200 gm. given there was an increase in the heat production of about 10 per cent. Thus, with 400 gm. the total increase was 38 per cent; with 600 gm. it was 51 per cent; with 800 gm. it was 60 per cent. It is clear that, while the specific dynamic action of the beef increased with each successive increase in the amount ingested, it was not true that the effect was a linear function of the amount ingested.

However, as previously shown by Lusk, if one estimates the amount of beef protein actually metabolized, the curve is more nearly parallel to that of the specific dynamic effect. Of the amount metabolized the best criterion is afforded by taking the nitrogen excretion at its peak, which occurs about the 5th hour after administering large quantities of beef. The reason for this has been frequently expounded. Using this as a comparable

TABLE V.
Influence of Increasing Quantities of Beef.

	200 gm.	400 gm.	600 gm.	800 gm.
Increase in total calories per hr.....	4.92	6.27	8.43	9.81
“ “ protein “ “ “.....	9.74	16.77	23.42	31.13
Metabolism of 100 protein calories causes increase in total calories.....	50	37.5	36	32

measure of the quantity of protein metabolized during the earlier hours, one obtains the results shown in Table V.

It is probable that the nitrogen elimination of the 5th hour after giving 200 gm. of meat may have been an improper expression of the protein metabolism of the 2nd to 5th hours following the ingestion of this small quantity of meat.

Turning to the older work of Williams, Riche, and Lusk (5) and recalculating the values according to the method above described, one finds, after giving 1,200 and 700 gm. of meat to Dog I that for each 100 calories liberated from extra protein metabolized during the 2nd to 5th hours, inclusive, there was an extra heat production, respectively, of 37 and 35 calories in total metabolism.

The relative values may therefore be summarized as in Table VI.

Calculated in the same fashion for Dog I, the hours next follow-

ing show somewhat higher values than those given above; *i.e.*, 44 calories of extra heat production for every 100 calories of increase in protein metabolism. It is probable that the true specific dynamic action is measured by the factor 45, as found for the 24 hour period by Williams, Riche, and Lusk, and that the lower figures during the 4 earlier hours are due to the fact that the protein metabolism of the 2nd and 3rd hours after meat ingestion cannot be measured by the nitrogen in the urine of the 5th hour, even though this constitutes a better measure of the protein metabolism of these early hours than does the nitrogen excretion of the hours themselves.

TABLE VI.

The Specific Dynamic Action of Meat during the 2nd to 5th Hours Following Its Ingestion.

Dog No.	Meat ingested.	Every 100 calories of protein metabolized increases total metabolism.
	<i>gm.</i>	<i>cal.</i>
XIX	200	50
XIX	400	37.5
XIX	600	36
I	700	37
XIX	800	32
I	1,200	35

It is clear that the specific dynamic action increases when the amount of protein metabolized increases, just as it increases when the amounts of glycine and alanine metabolized increase, but that if these amino acids are given with a protein, their specific dynamic effects may be neutralized.

We were able in these experiments incidentally to confirm the observations of Atkinson, Rapport, and Lusk (6), who found that after giving very large quantities of beef there was a conversion of protein into fat. In the present experiments, when 200 gm. of beef were administered, the total metabolism was 21.40 calories, the protein metabolism was 12.46 calories (as calculated from the urinary nitrogen), the respiratory quotient was 0.81 and the non-protein respiratory quotient was 0.82, the latter indicating that the non-protein metabolism was about 40 per cent of fat and 60 per cent of carbohydrate. When 400 gm. of beef were given the total metabolism was 22.75 calories, of which nearly all was protein (19.49 calories). The non-protein respiratory quotient was 0.91, indicating that of the

small non-protein metabolism, about 70 per cent was of carbohydrate origin. When 600 gm. of beef were given the number of calories that were theoretically available from the ingested protein was 26.14. Of these only 24.91 calories were metabolized, indicating a deposit of material in the body. This material, if oxidized, would have had a respiratory quotient of 0.82; that is, it was composed of about 40 per cent of fat and 60 per cent of carbohydrate. When 800 gm. of beef were given the deposit would have yielded, if oxidized, 7.56 calories, with a respiratory quotient of 0.72, indicating that the material deposited was entirely fat.

E. Ingestion of Glycine and Alanine Together.

Lusk (3) found that when glycine and alanine were given together there was a definite summation of effect. Thus, in a

TABLE VII.

The Ingestion of Beef and Casein Together.

(Average per hour of experimental period.)

Food.	Experiment No.	Date.	R. Q.	CO ₂	O ₂	Urinary N.	Calories.		Increase over basal.
							Indirect.	Direct.	
		1923		gm.	gm.	gm.			per cent
Beef, 200 gm. . .	Average of Nos. 98, 103, and 104.		0.83	7.62	6.72	0.350	22.05	22.40	33.8
Casein, 43.67 gm.	Average of Nos. 108 and 114.		0.80	7.25	6.55	0.277	21.54	21.42	30.7
Beef, 100 gm., and casein, 21.84 gm. . . .	131	Apr. 6	0.82	7.49	6.62	0.314	21.77	21.62	32.1

dog weighing about 11.5 kilos, 5.5 gm. of glycine raised the heat production 7.3 per cent; 5.5 gm. of alanine increased it 7 per cent; while the two mixed together raised it 18 per cent.

In the preliminary reports of this work it was stated that this result could not be confirmed. The statement was based on a single experiment in which 16.85 gm. of alanine showed an increase in metabolism of 29 per cent. On repeating the experiment we found increases in metabolism of only 23 and 19 per cent, respec-

R.Q.	0.80	0.81	0.80	0.82
—	16.48	22.05	21.54	21.77
----	16.86	22.35	21.42	21.62
% INCREASE				
OVER BASAL	33.80	30.67	32.10	
CAL.				
25 —	HEAT CALCULATED			
----	”	FOUND		

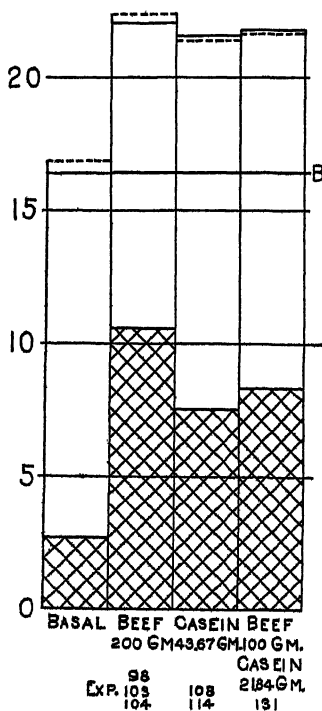


CHART IV. The effect of ingesting beef with casein.

tively, or an average of 21 per cent. Since 10 gm. of glycine also produced an increase of 21 per cent, and glycine and alanine together one of 32 per cent, it is evident that there is a profound summation of effect.

F. Ingestion of Beef and Casein Together.

The demonstrated power of casein and gelatin to neutralize the specific dynamic action of glycine suggested the possibility that two different proteins might neutralize each other. Does one partake of cheese after dining upon beefsteak because the amino acids of the former do not exercise a specific dynamic action but are neutralized by those of the latter? This led to investigating the behavior of two half portions of meat and contrasting the results with those obtained after giving full portions of either.

200 gm. of beef increased the heat production 34 per cent and 43.67 gm. of casein raised it 31 per cent. Upon giving half portions of each together, that is 100 gm. of beef and 21.84 gm. of casein, the rise in the heat production amounted to 32 per cent or as much as a full portion of each protein given alone would have induced. In other words, *when casein and meat protein are ingested together there is a summation of their specific dynamic action.*

G. The Factor of Intestinal Absorption.

1. *Comparison of the Metabolism of Gelatin, and of That of Glycine Given with Gelatin, during a Period of 7 Hours.*—It seemed possible that the neutralization of specific dynamic action of a protein and an amino acid when given together might be due to a delay in absorption of the ingested material from the intestine. To a certain extent the excretion of nitrogen might give us a clue to the solution of this problem. However, as before stated, the hourly rate of nitrogen excretion is a somewhat uncertain criterion of the rate of protein and amino acid metabolism. Moreover, this factor was too variable in our experiments to make it trustworthy. It seemed therefore desirable to follow the metabolism of a protein like gelatin during a longer period of time and to compare this with a similar record when gelatin and glycine were given together. If the lack of summation in the latter case were due to slower absorption, the specific dynamic action ought to be prolonged and the heat production be greater in the later hours than when protein alone was ingested. Table VIII shows the hourly metabolism through the 7th hour after the administration of gelatin and of gelatin and glycine together. The curve is

plotted in Chart V. There appears to be no significant alteration in the course of the specific dynamic action in the two experiments.

TABLE VIII.

The Hourly Metabolism after the Ingestion of Gelatin Alone and of Gelatin and Glycine Together.

Food.	Hrs. after food.	CO ₂	O ₂	R. Q.	Urinary nitrogen.		Calories.		Increase over basal.
					Average of calorimeter period.	Fractionated urine.*	Indirect.	Direct.	
		gm.	gm.		gm.	gm.			per cent
Gelatin, 38.74 gm.†	2	7.43	6.33	0.85	0.371	0.258	20.82	20.81	26.33
	3	7.74	6.47	0.87	0.371	0.392	21.41	21.73	29.92
	4	7.65	6.45	0.87	0.371	0.493	21.31	20.47	29.31
	5	7.64	6.64	0.85	0.371	0.560	21.80	21.76	32.28
	6	6.77	6.38	0.77	0.348	0.683	20.63	20.16	25.18
	7	6.87	5.97	0.84	0.348	0.694	19.60	21.22	18.93
	Average.....	7.35	6.37	0.84			20.93	21.03	27.00
Gelatin, 38.74 gm., and glycine, 10 gm.‡	2	7.72	6.77	0.83	0.391		22.18	20.59	34.59
	3	7.23	6.26	0.84	0.391		20.51	21.28	24.45
	4	7.73	6.09	0.92	0.391		20.23	21.37	22.75
	5	7.79	6.52	0.87	0.391		21.57	20.19	30.89
	6	7.08	6.22	0.83	0.746		20.08§	19.85	21.84
	7	7.12	6.11	0.85	0.746		20.19§	21.79	22.51
	Average.....	7.44	6.33	0.86			20.79	20.83	26.15

* This was obtained by giving the substance to be tested to the animal upon a day in which it was not put into the calorimeter.

† Quiet. 2nd to 5th hours, average of Experiments 101 and 141 (see Table VIII, previous paper); 6th and 7th hours, Experiment 100.

‡ 2nd to 5th hours, Experiment 118; 6th and 7th hours, Experiment 138.

§ Since the oxygen consumption was slightly less than would suffice to oxidize the protein metabolized, as indicated by the urinary nitrogen, this experiment was calculated upon the CO₂ (using Loewy's (7) factor of 1 liter of protein CO₂ = 5.579 calories).

2. *Parenteral Injections of Glycine with and without Oral Administration of Gelatin.*—Parenteral injections of glycine were

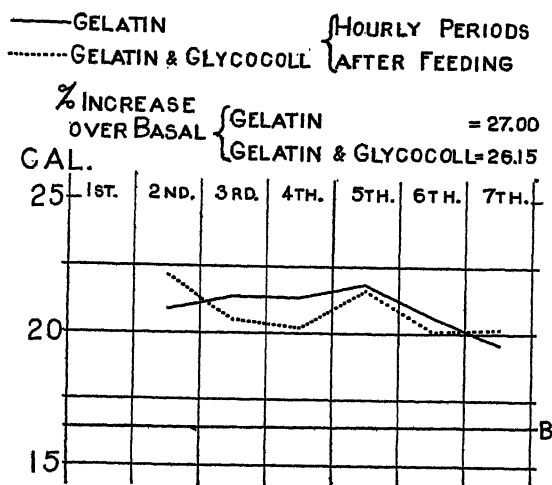


CHART V. A comparison of the metabolism through the 7th hour after ingesting gelatin, and gelatin plus glycine.

administered both subcutaneously and intravenously in order to establish the effect of these materials during the same interval of time as that in which gelatin was being absorbed by the intestinal tract. This would indicate whether gelatin exerted its neutralizing effect in the gastrointestinal tract or in the tissues themselves.

Krzywanek (8) has reported extraordinary and irregular increases in the metabolism of dogs after administering glycine intravenously. Thus, in a dog weighing 7.3 kilos the intravenous injection of 5.4 gm. of glycine increased the heat production 107 per cent at the end of 30 minutes and 72 per cent at the end of an hour. These astonishing results could not be confirmed.

When given subcutaneously, 10 gm. of glycine were first dissolved in 60 cc. of distilled water and injected at body temperature into the back near the vertebral column; when given intravenously the same amount and volume of material were injected into the external jugular vein, the duration of the injection being between $1\frac{1}{2}$ and 2 minutes. In neither case did the animal evince any distress. During and following the intravenous injection a slight quickening of respiration was sometimes observed.

TABLE IX.
Experiments Involving Parenteral Injections of Glycine.

(Average per hour of experimental period.)

Food.	Experiment No.	Date.	R. Q.	CO ₂	O ₂	Urinary N.	Calories.		In-crease over basal.
							Indirect.	Direct.	
		1924		gm.	gm.	gm.			per cent
Glycine (by mouth), 10 gm.....	119, 170, 176		0.87	7.15	5.99	0.310	20.01	19.29	21.42
" (subcutaneous), 10 gm.....	177	Mar. 8	0.80	6.35	5.77	0.255	18.96	17.68	15.05
" (intravenously), 10 "	183, 191		0.86	6.94	5.88	0.306	19.55	18.42	18.62
Gelatin, 40.4 gm.....	169, 178		0.87	7.74	6.47	0.422	21.33	19.66	29.42
" 40.4 " glycine (subcutaneous), 10 gm..	168, 179		0.87	7.82	6.52	0.400	21.55	21.58	30.76
" 40.4 " " (intravenously), 10 " ..	180, 193, 195		0.86	7.77	6.41	0.492	21.49	20.80	30.40

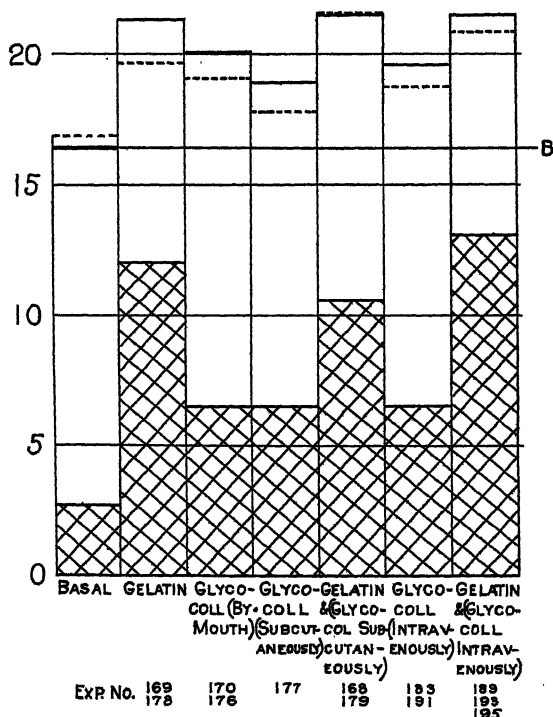
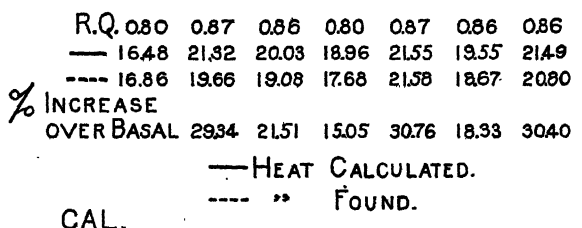


CHART VI. The effect of parenteral injections of glycine.

In the earlier experiments gelatin gave an increase in metabolism of 29 per cent. For purposes of comparison these experiments were repeated a year later and an average increase of 29 per cent was obtained. It appears phenomenal that during a period of a year the basal metabolism remained constant and the body mass reacted with quantitative exactness to the stimulus induced by the administration of 39 gm. of gelatin.

10 gm. of glycine, injected subcutaneously, caused the metabolism to rise 15 per cent. When this subcutaneous injection of glycine was combined with the administration of gelatin by mouth the metabolism rose 31 per cent (Table IX), or no greater than the increase produced by gelatin alone.

Similar results occurred when 10 gm. of glycine were injected intravenously and when this treatment was followed immediately by the ingestion of gelatin. The intravenous injection produced an increase in the metabolism of 19 per cent, and when this was combined with the administration of gelatin *per os*, the increase in the metabolism was 30 per cent. Again the lack of summation is obvious.

It is clear, therefore, that the complete absence of summation when gelatin and glycine were given together by mouth was not due merely to a delay in the absorption of the material from the gastrointestinal tract. Whatever the reactions are that are responsible for the suppression of the specific dynamic action of these substances when administered together, they may take place independently of the intestinal tract.

The increases in heat production between the 2nd and 5th hours after administering 10 gm. of glycine by different pathways to Dog XIX seem quite comparable (Table IX).

Food.	Increase above the basal metabolism.
	<i>per cent</i>
10 gm. glycine, <i>per os</i>	21
10 " " subcutaneously.....	15
10 " " intravenously.....	19

Little difference is to be observed.

On account of the results obtained by Krzywanek shortly after intravenous injection of glycine, two metabolism observations of the second half of the 1st hour after the injections were made on Dog XIX with results which showed no increase in one case and one of 10 per cent in another. The heat production was measured from the oxygen consumption, for during this short period the respiratory quotient was 1.25, indicating a neutralization of alkaline carbonate by the glycine. The results may be thus epitomized:

Experiment No.	Time.	R. Q.	CO ₂	O ₂	Calories.		Increase over basal.
					Indirect.	Direct.	
			gm.	gm.			per cent
183	2nd half hr.	1.25	3.82	2.22	7.48	8.8	-9
	2nd hr.	0.81	7.89	7.06	23.25	18.7	42
191	2nd half hr.	1.15	4.21	2.67	9.06	11.0	10
	2nd hr.	0.88	7.58	6.27	20.93	19.5	26

The research terminates only with a query. Is it possible for the amino acid, glycine, for example, to be chemically united with the products of gelatin or of casein hydrolysis so that it does not follow the same pathway in metabolism as it does when given alone? The possibility of difference in biological reaction of combined and uncombined proteins has lately been shown in Kossel's laboratory by Felix and Morinaka (9) who demonstrated by liver perfusion that arginine was freely split into urea and ornithine, whereas arginine in peptide form was not destroyed by the liver.

A possible method suggested itself from these reflections. Would asparagine, which is practically devoid of specific dynamic action (10), neutralize the effect of glycine?

H. Ingestion of Asparagine and Glycine Together.

Asparagine, being insoluble in water, was administered by mixing it with lard, a mixture which the dog ate readily (Table X). 10 gm. of lard increased the metabolism 13 per cent; when asparagine was mixed with lard the resultant rise in metabolism was slightly, but not significantly, higher; namely, 18 per cent. Glycine, mixed with lard in a similar manner, raised the metabolism 29 per cent, while the mixture of glycine, asparagine, and lard resulted in an increase of 25 per cent. While this is slightly lower than the resultant rise in metabolism when glycine and lard were given, the difference is too small to be significant, and we cannot say that the ingestion of asparagine with glycine has an appreciable effect on the specific dynamic action of the latter.

Though the search may be long and wearisome, yet it is possible that some day an amino acid or some polypeptide group may be found which, when ingested with glycine, is capable of neutralizing its specific dynamic action.

TABLE X.
The Effect of Giving Asparagine and Glycine Together.
 (Average per hour during 4 hourly periods.)

Food.	Experiment No.	Date.	R. Q.	CO ₂	O ₂	Urinary N.	Calories.		In-crease over basal.
							Indirect.	Direct.	
		1924		gm.	gm.	gm.			per cent
basal.....			0.82	5.57	4.97	0.103	16.48	16.86	
lard, 10 gm.....	186	Apr.	3 0.81	6.23	5.59	0.116	18.61	17.93	13.0
lycine, 10 gm.....	119, 170, 176					0.310	20.01	19.29	21.4
" 10 " and lard, 10 gm.....	188	Apr.	5 0.87	7.15	5.99	0.306	21.21	20.22	28.7
asparagine, 15 gm., and lard, 10 gm.....	184, 187					0.256	19.43	18.37	17.9
" 15 " glycine, 10 gm., and lard, 10 gm..	182	Mar.	28 0.91	7.77	6.23	0.424	20.59	19.59	24.9

I. The Nitrogen Elimination.

Table XI summarizes the figures of the urinary nitrogen elimination.

TABLE XI.
The Nitrogen Elimination.

No. of experiments.	Diet.	Urine collection after food.	Nitrogen per hr.
		hrs.	gm.
18	Basal.	18 to 21	0.10
1	Lard, 10 gm.	5	0.12
1	Beef, 100 gm.	5½	0.23
2	Asparagine, 15 gm., lard, 10 gm.	5½	0.26
1	Glycine, 10 gm. (subcutaneously).	5½	0.26
1	Casein, 21.84 gm., beef, 100 gm.	5½	0.31
3	Glycine, 10 gm.	5½	0.31
2	Glycine, 10 gm. (intravenously).	5½	0.31
1	" 10 " lard, 10 gm.	5½	0.31
2	Alanine, 16.85 gm.	5½	0.35
1	Gelatin, 37.84 "	6th and 7th	0.35
1	Casein, 43.67 gm., alanine, 16.85 gm.	5½	0.37
1	" 43.67 " glycine, 10 "	5½	0.38
1	Gelatin, 38.74 " " 10 "	5½	0.39
2	Glycine, 10 gm. (subcutaneously), gelatin, 40.4 gm.	5½	0.40
2	Gelatin, 40.4 gm.	5½	0.42
1	Asparagine, 15 gm., lard, 10 gm., glycine, 10 gm.	5½	0.42
1	Gelatin, 38.74 gm., glycine, 10 gm., alanine, 16.85 gm.	5	0.43
1	Gelatin, 38.74 gm., alanine, 16.85 gm.	5½	0.45
1	Glycine, 20 gm.	5½	0.46
3	Glycine, 10 gm. (intravenously), gelatin, 40.4 gm.	5½	0.50
2	Alanine, 16.85 gm., glycine 10 gm.	6	0.53
1	Gelatin, 38.74 " " 20 "	6½	0.58
1	" 38.74 " " 10 "	6th and 7th	0.75

The first impression on considering these figures is that, when an amino acid is administered with a protein, the amino acid is in some way combined in the body and, therefore, is not metabolized. However, in the experiments that were continued through the 6th and 7th hours the nitrogen elimination rose from

0.35 gm. after the ingestion of gelatin alone to 0.74 gm. when gelatin and glycine were ingested, yet the calorimetric study of these hours showed no greater increase of metabolism after the administration of gelatin and glycine than after gelatin alone, notwithstanding the evident destruction of the amino acid.

We are unable at the present time to advance a satisfactory explanation for these contradictions. Hence, the nitrogen elimination in this series of experiments fails to throw light upon the reasons for the summation or inhibition, as the case may be, of specific dynamic effect which we have considered. This is probably because the hourly nitrogen elimination is an untrustworthy index of the true protein metabolism in the experimental periods and, more particularly, of the metabolism of the individual amino acids.

J. Relation to Former Work.

At the International Congress on Hygiene and Demography, held in Washington in 1912, Lusk (11) differentiated between (1) *a basal metabolism*, (2) *a metabolism of plethora* due to an inflow into the blood of an excessive quantity of carbohydrate or fat, and (3) *a metabolism of amino acid stimulation* as induced especially by glycine and alanine. He found that after adding glycine to a mixed diet consisting of biscuit meal, a little fat, and a small amount of meat there was no increase in the heat production above that produced by the ingestion of the same diet without glycine. He concluded: "the metabolism of plethora and the metabolism of amino-acid stimulation cannot be added to each other; there is no summation of effect when both influences are brought into action together."

3 years later Lusk (12) announced that "after giving glucose, 50 gm., with glycine, 20 gm., the increase in the metabolism was almost as great as the sum of the increases induced when each substance was given alone. Alanine, 20 gm., followed the same law when given with glucose, 50 gm." And he added: "This nullifies the author's former opinion."

These relations were extended to the behavior of fat by Murlir and Lusk (13) and seemed to be in direct contradiction to the earlier work.

In view of the facts set forth in the present paper, it appears probable that the quantity of protein given in the mixed diet was sufficient to neutralize the effect of the glycine or alanine added to it.

K. Alcohol Checks.

The validity of the experimental data that we obtained is confirmed by the results of alcohol checks made at intervals during the course of the work (Table XII; see also previous paper).

TABLE XII.
Alcohol Checks.

Experiment No.	Date.	R. Q.	Calories per hr.		Difference.
			Indirect.	Direct.	
	<i>1923</i>				<i>cal.</i>
182	Nov. 19	0.667	23.08	22.86	-0.22
183	Dec. 26	0.667	20.72	20.91	+0.19
	<i>1924</i>				
184	Jan. 10	0.664	16.12	15.95	-0.17
185	Feb. 25	0.670	16.82	17.02	+0.20
186	Mar. 21	0.662	17.15	18.11	+0.96
188	Apr. 10	0.666	22.96	22.28	-0.68
Average.....		0.666	19.47	19.52	+0.05

In order to avoid a considerable expense the detailed tables of the extensive material presented in this paper have been voluntarily suppressed. The full records, however, are on file in this laboratory.

IV. SUMMARY.

1. On the theory that glycine and alanine were the chief factors contributing to the specific dynamic action of protein, it was possible to postulate that the specific dynamic action of those proteins which contain little or no glycine or alanine might be due to the synthetic production of one or the other of these amino acids when such proteins were ingested. This would explain the fact that the proteins studied had the same heat-stimulating properties. When casein, which contains no glycine, was given with glycine, there was no greater specific dynamic action than when casein was given alone, which seemed to indicate

a possible inhibition of the synthesis of this amino acid when it was ingested (see following paper by Csonka). However, when 10 gm. of glycine were given with 39 gm. of gelatin (6 gm. of N), which itself yields on hydrolysis 10 gm. of glycine, there was again no greater increase in the heat production than when gelatin was given alone. The specific dynamic action of glycine was neutralized.

2. When the quantity of gelatin given with glycine was reduced to 10 gm., the metabolism was greater than would have been induced by either substance alone—a summation of effect.

3. When alanine was given with casein or with gelatin there was no summation of effect, the heat production being the same as when either of these proteins was administered alone.

4. When the quantity of glycine given with gelatin was increased to 20 gm. the specific dynamic action was also increased and there was again a summation of effect.

5. When increasing quantities of beef were given there was a corresponding increase in the specific dynamic action. In general the increase was directly proportional to the amount of protein metabolized.

6. When glycine and alanine were given together the specific dynamic action was much greater than when one of them was fed alone.

7. When glycine and alanine were given with gelatin the specific dynamic action was practically the same as when gelatin alone was ingested.

8. When the proteins, meat and casein, were administered together there was a complete summation of their specific dynamic action. One does not take cheese after meat in order that the heat-stimulating value of the latter may be neutralized.

9. When gelatin and glycine are given together and the metabolism is compared with that following the ingestion of gelatin alone during a period of 7 successive hours it is found that the two curves produced from the data are virtually the same. One cannot, therefore, explain the absence of summation as due to a delay in intestinal absorption.

10. When the ingestion of gelatin is combined with either subcutaneous or intravenous injection of glycine, the specific dynamic action is no greater than when gelatin is given alone. It

seems, therefore, that the reactions which neutralize the effect of the amino acids may occur in the blood or in the tissues outside of the gastrointestinal tract.

11. The specific dynamic action of glycine is not appreciably different whether it is given by mouth, subcutaneously, or intravenously.

12. The increase in metabolism after giving asparagine and glycine together is not significantly different from the increase after giving glycine alone. The specific dynamic action of glycine is not neutralized therewith.

13. It is probable that gelatin and casein yield certain amino acids or polypeptides which, reacting with glycine, neutralize its specific dynamic effect.

14. From the foregoing facts, we believe that other important factors, as yet unknown, contribute to or modify the specific dynamic action of protein, apart from the direct influences of the amino acids, glycine and alanine.

15. Incidentally, it was confirmed that when a large amount of meat is ingested there is a retention in the body of a pabulum, derived from the end-products of protein metabolism, which consists partly of carbohydrate and partly of fat, and that upon the ingestion of a still larger amount of meat there is a deposit of fat alone.

We wish to express our indebtedness to Professor Graham Lusk for his constant and valued aid.

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ANIMAL CALORIMETRY.

TWENTY-SEVENTH PAPER.

ON THE ADMINISTRATION OF VARIOUS PROTEINS WITH BENZOIC ACID TO A PIG.*

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*The data in this paper are taken from a dissertation presented by F. A. Csonka to the faculty of the Graduate School in Cornell University, 1924, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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I. INTRODUCTION.

The researches of Lusk and his collaborators (1) show that the ingestion of glycine, as well as alanine, in the normal and also in the phlorhizinized dog raises the heat production above the level of the basal metabolism and that the increase of heat output is slight when other amino acids present in native proteins are ingested. These findings would suggest that the glycine and alanine present in protein are chiefly responsible for the increased heat elimination above the basal level; *i.e.*, the specific dynamic action of protein. However, the specific dynamic action as observed by Rubner after meat ingestion is higher than that when gelatin is administered, although meat protein contains only 4 per cent, while gelatin contains 25 per cent of glycine. The content of alanine in both of these proteins is about the same. Casein, which does not contain any glycine, shows a specific dynamic action similar to that of gelatin.

The question arises whether protein metabolism *in vivo* produces glycine and alanine in the same amount as that obtained on acid hydrolysis *in vitro*. The great similarity in the intensity of the specific dynamic action of different proteins might be due to a large synthetic production of glycine from the proteins which contain no preformed glycine, or the results might be due to other unknown factors that differentiate protein metabolism from that of pure amino acids.

When benzoic acid is administered¹ to an animal it is largely combined with glycine and eliminated as hippuric acid. Glycine is the only amino acid which, when given together with benzoic acid, will increase the quantity of hippuric acid elimination. The quantity of hippuric acid formed might therefore serve as a clue to the amount of glycine produced as such during protein metabolism.

The purpose of the investigation reported here is to make a quantitative study of the hippuric acid production when proteins of

¹ Following the usual conventional form, values are given in benzoic acid, although it should be understood that sodium benzoate was given to the animal.

different varieties, with different glycine content, are fed to a pig which is at the same time under the influence of benzoic acid, and to note whether or not the quantity of hippuric acid formed is influenced by the preformed glycine present in the food; *i.e.*, the protein under investigation.

II. HISTORICAL.

1. Glycine Synthesis in the Animal Body.

Glycine is a normal constituent of body protein, 4 per cent of it being present in the muscle protein, as shown by Osborne (2). The new-born baby lives on a practically glycine-free diet (milk) and yet increases its weight, and the newly developed tissue contains 4 per cent of glycine which is not supplied by the food.

Willcock and Hopkins (3) showed that the presence of glycine in the food proteins is not required for maintenance or for growth, since mice fed with a glycine-free diet in which casein supplied the sole protein lived and showed normal growth. When zein, which is free from glycine, tryptophane, and lysine, was substituted for casein, and the last two missing amino acids were added, Osborne and Mendel (4) found that the rats did not suffer on account of the absence of glycine but they developed normally in every respect.

All these facts demonstrate a synthetic production of glycine in the normal animal.

When benzoic acid is given to an animal it is eliminated in the urine largely as hippuric acid.

Parker and Lusk (5) were the first to investigate the elimination of hippuric acid in rabbits under the influence of benzoic acid when different proteins were given. They reported that the hippuric acid formation was not influenced by casein or gelatin. Cohn (6), however, observed an increase of hippuric acid output in the rabbit when gelatin was administered with benzoic acid, while no change occurred when the diet contained casein. Lewinski (7), in his study on the limits of hippuric acid formation in man, noted that the same quantities of benzoic acid (40 gm.) induced a lower hippuric acid elimination on a low protein diet than on a high protein diet consisting of albuminoids. The high glycine content of the latter explains his results.

Raiziss and Dubin (8) observed an increased hippuric acid excretion on a milk and egg diet and concluded that a high protein level of metabolism favored the synthesis of hippuric acid.

During the course of my investigation the work of Griffith and Lewis (9) was published. They were particularly interested in the rate of hippuric acid formation as influenced by different proteins. The proteins were hydrolyzed *in vitro* with pepsin followed by trypsin to eliminate digestive factors, which might have influenced the rate of absorption, although

unhydrolyzed proteins were also tested. When these were fed to rabbits it was found that the rate of synthesis of hippuric acid was markedly increased when proteins with a high glycine content were ingested, *i.e.* gelatin and elastin, while proteins like casein and egg albumen, which are lacking in glycine, were without effect on the rate of hippuric acid synthesis.

The results of these investigations point to two available sources for the origin of glycine in hippuric acid formation; first by synthesis, and second from preformed glycine. The latter one includes the glycine liberated by the catabolism of body protein and that present in the food protein.

2. Elevated Protein Metabolism Following Benzoic Acid Administration.

Salkowski (10) first noted an increase of urinary nitrogen in the rabbit after the administration of benzoic acid. As a result of additional experiments carried out on dogs he (11) later attributed this increase to the nutritive condition of the animal, since those animals which were capable of detoxicating benzoic acid by the formation of hippuric acid showed a small increase or none at all in nitrogen excretion above normal figures. Both Pribram (12) and Wiechowski (13) found an increased urinary nitrogen after the ingestion of benzoic acid as well as after giving hippuric acid. Magnus-Levy (14) also observed an increase of nitrogen elimination in the urine of sheep. On the 1st and 2nd days of starvation there were 5 to 7 gm. of nitrogen as compared to 11 to 13 gm. when large quantities of benzoic acid were administered. Ringer (15) reported an increase of urinary nitrogen in the case of a goat after large amounts of benzoic acid were given, without changing the quantity of urea eliminated, however, and he thought that the glycine originated from the extra protein catabolized, as represented by the increased urinary nitrogen noted. Delprat and Whipple's experiments (16) on starving dogs, when benzoic acid was directly injected into the circulation, showed a definite increase of urinary nitrogen.

McCollum and Hoagland (17), on the other hand, found practically no change in the urinary nitrogen elimination after feeding benzoic acid to a pig, which is hard to explain unless this animal represents an exception. However, their animals were not starving but were maintained with a pure starch diet, which is known to have a strong sparing action on the body protein.

Shiple and Sherwin (18) came to the same conclusion as McCollum and Hoagland; namely, that benzoic acid, when fed in moderate doses (3 to 10 gm.) to man, does not increase the nitrogen metabolism when the subject has been reduced practically to a state of endogenous protein catabolism.

3. The Appearance of a Reducing Substance in the Urine When Benzoic Acid Is Administered.

In 1880 Salkowski (19) first reported the presence of a reducing substance in the urine of dogs, rabbits, and men when they were under the influence of

benzoic acid. Kobert (20) suspected that the reducing compound was a glycuronate, although this was purely a speculation, not based on any analytical results. Siebert (21) tried to identify this reducing material but was unable to separate it from dog urine. However, after hydrolyzing the urine with sulfuric acid, he succeeded in showing the presence of glycuronic acid. Magnus-Levy (22) reported that the reducing substance, which he isolated from the urine of sheep maintained under the influence of benzoic acid, was a benzoic ester of glycuronic acid, dextro-rotatory and unfermentable. In the urine of dogs after benzoic acid ingestion Brugsch and Hirsch (23) noted a dextro-rotatory compound and expressed their belief that it was very likely identical with the substance found by Magnus-Levy. The compound isolated later by Magnus-Levy is as yet the only glycuronate which is dextro-rotatory, all the others known being levo-rotatory. It is very soluble in water, showing an acid reaction, and up to the present time has not been prepared in crystalline form.

Magnus-Levy's technique, as he himself admits, is somewhat cumbersome. However, he was able to prepare a crystalline strychnine compound of the benzoyl glycuronic acid which enabled him to prove the identity of the urinary constituent as a glycuronic ester of benzoic acid. Whether this is the only substance which is responsible for the strong reduction is yet unknown.

4. Toxic Effect Caused by the Ingestion of Benzoic Acid.

Meissner and Shepard (24) in 1866 reported that some individuals taking 5 to 7.6 gm. of benzoic acid after breakfast experienced nausea and vomiting, while others showed no toxic effect. These investigators gave 8 gm. of benzoic acid twice daily to dogs and noted convulsions, spasms, and foam at the mouth.

Kobert (20) observed toxic symptoms in cold-blooded animals after the administration of benzoic acid, manifested by clonic spasms and vomiting. In warm-blooded animals (rabbits, cats, and dogs) toxic doses produced tremors, convulsions, and dullness, followed by incoordinated movements of the fore legs and a progressive paralysis from the anterior extremities backwards. Coincident with these nervous disturbances there was a fall in body temperature. Death occurs from paralysis of the respiratory center.

Lewipiski (7) succeeded in avoiding these toxic effects in men by administering the benzoic acid in small doses and at the same time prescribing for them a high protein diet. Under these precautions he was able to introduce 40 gm. of benzoate daily without ill effects.

Since benzoic acid is commonly used as a preservative in canned food-stuffs, it was important to determine whether or not the amount applied for that purpose should be limited. Chittenden, Long, and Herter (25), appointed by the United States Food Commission, carried out an extensive investigation of its toxicity, with the following conclusion: Small quantities up to 4 gm. daily in healthy young men caused no ill effects or toxic symptoms, therefore the curtailment of its use within these limits is uncalled for.

There is no reference found in the literature in which toxic symptoms have been observed in pigs under the influence of benzoic acid, although McCollum and Hoagland (17) and also Abderhalden and Strauss (26) administered comparatively large doses of it to these animals.

5. *Comment.*

One of the most confusing causes giving ground for contradictory statements in the literature is the failure to emphasize the species of animal on which the experiments were carried out, since animals of different species react differently to benzoic acid. Rabbits and dogs have been the animals most frequently used in the past. The former eliminate ingested benzoic acid in the form of hippuric acid, and glycuronate is said to be present only in traces, while the dog eliminates less than 50 per cent of the benzoic acid as hippuric acid and the rest appears partly free and partly in conjugation with glycuronic acid.

Pigs were used with benzoic acid ingestion in investigations carried out by McCollum and Hoagland (17) and also by Abderhalden and Strauss (26), but none of these investigators noted the presence of the reducing glycuronate compound in the urine.

The identity of the reducing substance, *i.e.* benzoic ester of glycuronic acid, was established in 1907 by Magnus-Levy (22), yet this form of conjugated benzoic acid has not been considered in the experiments carried out since then. The assumption has been that in certain species this reducing compound does not occur in sufficient quantities to interfere with the reliability of the hippuric acid determination. By overlooking the presence of the reducing substance, erroneous conclusions might be drawn, especially when the determination of the hippuric acid is based upon the difference in the amount of total and free benzoic acid. Recently, Kingsbury and Bell (27) have reported experiments on nephrectomized dogs similar to those of Bunge and Schmiedeberg (28) and have found hippuric acid formation by analyzing the animals' blood. They concluded, therefore, that the kidneys are not the only organs in which the hippuric acid synthesis might take place. It is not certain, however, that the experimental proof brought forward by Kingsbury and Bell is clear-cut because they determined the amount of conjugated benzoic acid which, especially in the case of dogs after benzoic acid administration, is largely composed of glycuronic ester. What they estimated

in the blood, therefore, was not necessarily entirely attributable to a conjugation with glycine; *i.e.*, hippuric acid. The older methods, in which a direct isolation of hippuric acid served as a basis for its quantitative determination, were unreliable. Most of them required evaporation of the urine to a small volume or even to dryness to avoid losses through solubility, a procedure which may cause some hydrolysis of hippuric acid into its components. Furthermore, the purity of the hippuric acid so obtained is questionable.

Some of the earlier workers did not take precautions regarding the preservation of the urine or they used alkali for that purpose, in which case, as has been pointed out by Folin and Flander (29), even more favorable conditions for the splitting of the hippuric acid were produced.

If the quantity of benzoic acid administered is too small, the variation in the amount of hippuric acid eliminated may fall within the limit of experimental error; on the other hand, too large a dose of benzoic acid may produce toxic symptoms, and then one deals with pathological rather than with normal conditions.

The rate expressed in the percentage of total benzoic acid eliminated should be calculated from the amount ingested, but the proportion of combined and free benzoic acid should be based on the total eliminated, because when large amounts are administered there may be a temporary retention of benzoic acid, which may obscure the actual relationship of these two forms.

III. EXPERIMENTAL PART.

1. *General Plan.*

All the experiments reported in this paper were carried out on one male Berkshire pig, 8 weeks old at the beginning of the work and apparently a perfectly normal animal. After the pig became accustomed to the special food which was used it grew quite tame, so that it was no hardship to handle it even when it had reached the age of 8 months and weighed over 30 kilos. The nutrition cage, specially designed and recommended by E. V. McCollum and H. Steenbock (30), was adopted and found to be practical. When benzoic acid ingestion was commenced the

food was taken voluntarily only on the 1st day, and thereafter the benzoic acid, as well as the diet to be tested, was administered by a stomach tube. This procedure required the aid of three persons: one to hold the pig in an upright position, another to keep the mouth gag firmly in place, and the third one to introduce the stomach tube and to pour the liquid food through it. It impresses one as a rather cumbersome manipulation, but after a short time it proved to be a simple, quick, and by all means the most reliable technique. By direct feeding one cannot avoid a considerable loss by spattering.

A pig was selected since, as McCollum (31) has pointed out, its nutritional conditions are closely similar to those of man, both being omnivorous. Likewise, in feeding experiments in which varieties of proteins are the subject of investigation the pig is a more suitable animal than dogs or rabbits. Furthermore, the large volume of urine allows one to carry out an extended study of the various urinary constituents which, in the case of hippuric acid determination, is an especially important factor to take into consideration.

The urine was collected daily, and it was noted, although the separation of the 24 hour specimens was not obtained by the more reliable method of catheterization, yet, judging from the analytical data, it was surprisingly accurate. As an explanation of the reliability in the separation, it should be noted that the 24 hour period always ended at 10 a.m., because experience taught us that the pig usually urinated shortly before that time. Our main object was to follow the hippuric acid elimination and, since 8 gm. of benzoic acid were given twice daily at about 10 a.m. and 5 p.m., there was ample time for the complete elimination of the hippuric acid formed from the second dose before the morning of the following day. When benzoic acid was administered three times daily the dosage took place, at 10 a.m., 1 p.m., and 5 p.m. To minimize the unavoidable, although slight error caused by this method of 24 hour period separation, the experiments with each protein occupied 3 consecutive days.

It is very important to insure an acid reaction of the urine to avoid bacterial decomposition of hippuric acid, as pointed out by Raiziss and Dubin (8), and, therefore, the urine was collected in a bottle containing 100 cc. of a 1.5 to 2.0 per cent solution of sulfuric acid. Raiziss and Dubin recommended nitric acid for this purpose, but in order that the determinations of nitrogenous products should not be invalidated by the addition of nitric acid, the use of sulfuric acid was preferred.

It was noted after benzoic acid ingestion that in some specimens of urine, especially when preformed glycine was present in the food, hippuric

acid crystals separated out from the acidified urine. When such was the case the whole 24 hour specimen was filtered through a piece of cotton and this, with the crystals, washed from the other urinary constituents, was transferred to hot distilled water, which dissolved the hippuric acid so that it could be mixed with the urine for analysis.

2. *The Selection of the Diet.*

McCollum (31) showed that a pig can be kept for a long period of time with no disturbance, such as loss of appetite and loss of weight, when given a diet of starch, salt, and water, one which is practically free from protein. This dietary behavior gave an additional reason for selecting the pig as a subject for these experiments. The study of the effect of varieties of proteins on the hippuric acid formation required the selection of a protein-free diet to which the protein under investigation could be added.

Since the pig used in this investigation was a young, growing animal, the diet had to supply ample calories to cover, not only maintenance, but also the energy requirement expressed in the growth quota. To overcome the deficiency inherent in synthetic food mixtures when used as a diet over long periods, a water-soluble vitamin B preparation was given daily with the food. In addition to salt mixture used by Mendel and Karr (32) we added bone ash to insure semihard feces and so to avoid a contamination of the urine with fecal material.

The standard diet consisted of 200 gm. of corn-starch,² 4 to 5 gm. each of salt mixture and bone ash, 0.75 gm. of vitamin B (Harris' yeast vitamin powder), and water sufficient to make a milky mixture. To this diet we added protein containing 6.12 gm. of nitrogen, more or less, according to the nature of the question to be investigated.

The standard diet represents 820 calories. The average weight of the pig in the first series of experiments was 16 kilos, therefore the standard diet supplied 51 calories per kilo. When the calories derived from the protein were added to this, it insured an adequate calorific requirement for a growing animal. The basal metabolism of the pig was determined in a respiration calorimeter and was found to be 28 to 29 calories per hour. The calorific value of the standard diet plus protein was therefore more than sufficient for the needs of the animal, although intentionally not adequate for maximal growth. The pig was given the standard diet twice daily and water *ad libitum*.

We chose three proteins, casein, meat protein (in the form of meat powder), and gelatin. Each represents a certain type: casein in its relation to growth and maintenance; meat in its close relationship to body protein proper; and finally gelatin (33), characterized by its high glycine content.

²In the second and third series of experiments the amount of corn-starch was raised to 300 and 400 gm., respectively, to conform with the gain in weight of the pig.

The nitrogen content of the proteins used in these investigations was the following.

	per cent
Casein, technical (Merck).....	12.35
Casein, c. p. (Harris).....	13.35
Gelatin (Difco).....	14.85
Meat powder, beef ³	11.75
“ beef.....	3.61

In two instances chemically pure casein was substituted for the technical casein, but the results showed no difference in the hippuric acid production.

3. Analytical Methods.

a. Urine.—The total nitrogen in the urine was determined by the Kjeldahl method; for the determination of urea and ammonia Marshall's urease and Folin's ammonia methods, as modified by Van Slyke and Cullen (34) were used. The creatinine was determined colorimetrically according to Folin (35). The reducing power of the urine was estimated by the Benedict (36) quantitative method for sugar; the relevancy of this with regard to calculating the amount of urinary hippuric acid will be discussed later.

For determining free benzoic acid in the urine the method of Raiziss and Dubin (37) was employed. The total benzoic acid was determined by the modification of the Folin-Flander method described by Kingsbury and Swanson (38).

The difference between total and free benzoic acid gives the amount of conjugated benzoic acid in the urine, which includes both that combined with glycine (hippuric acid) and that with glycuronic acid (benzoyl glycuronic acid). The latter was estimated from its reducing property toward Benedict's quantitative reagent. Both glucose and glycuronic acid contain an aldehyde group which is the active radicle causing a reduction of the cupric to a cuprous compound. There is no reason to doubt that their reducing behavior toward copper is similar, and that the error introduced, when we determine and calculate the amount of glycuronic acid on the basis of glucose, is slight (39). To correct the differences between the two substances due to their molecular weight the reducing power expressed as glucose, obtained by the Benedict method, is multiplied by the factor

$$\frac{194}{180} = 1.077$$

An example will clearly demonstrate the method of calculation suggested:

It was found that 8.45 cc. of urine reduced 25 cc. of Benedict's quantitative copper solution, which by previous determination was found to represent 50.4 mg. of glucose. The glycuronic acid in terms of glucose for the urine of a 24 hour period (1,360 cc.) was 8.11 gm.; therefore, $8.11 \times 1.077 = 8.734$ gm. of glycuronic acid present in the urine. Since each mole-

³The dried meat powder was kindly furnished by Dr. G. McKee of Columbia University.

cule of benzoyl glycuronic acid contains 1 molecule of benzoic acid, therefore, $\frac{122.09 \times 8.734}{194} = 5.496$ gm. of benzoic acid existing in the form of gly-

curonic ester, which when subtracted from the "combined" figure (total benzoic acid minus free) gives the amount of benzoic acid combined with glycine (hippuric acid). 1 gm. of hippuric acid contains 0.6315 gm. of benzoic acid; the amount found by the above calculation divided by 0.6815 gives the quantity of hippuric acid present in the urine.

The preparation of benzoyl glycuronic acid from the urine is difficult because of its being a very unstable and amorphous compound; furthermore, it occurs with hippuric acid, from which it is difficult to separate it. Likewise, there is danger of hydrolysis by which the benzoic acid radicle is split off. Consequently, one would expect to find hippuric acid and free benzoic acid as impurities in a preparation of benzoyl glycuronic acid.

The method for the preparation of benzoyl glycuronic acid proposed by Magnus-Levy (22) was applied with some modifications. The urine was collected from the pig during benzoate administration, 9.5 liters of which showed a reduction equal to 37.2 gm. of glucose, and this served for the preparation. Ba (OH)₂ was added in an amount sufficient to precipitate the sulfuric acid, which had prevented bacterial decomposition during storage, and then a saturated solution of lead acetate until no more precipitate formed. The clear filtrate, acid in reaction, was evaporated to a volume of 1,100 cc. in vacuum at a temperature never higher than 50°C., usually at 45°. By this procedure practically no loss of the reducing substance was observed.

The glycuronate was precipitated by the addition of a saturated solution of basic lead acetate, filtered to remove other constituents, washed by suspending the lead precipitate twice in 300 cc. of distilled water, and, after shaking, filtered by suction. The filtrates from the lead precipitate showed a reduction representing 9.7 gm. of glucose, but in an attempt to concentrate the filtrate by evaporation the reducing substance was largely destroyed.

The lead precipitate was suspended in a liter of distilled water and H₂S was passed through until saturation. The PbS was filtered by suction and again suspended in 400 cc. of water and saturated with H₂S. The combined filtrates were concentrated to 1,240 cc., mainly to drive off the H₂S and make a quantitative determination of its reducing power possible. The reduction represented 17.1 gm. of glucose, showing that the loss of the benzoyl glycuronate in its preparation from the urine occurs chiefly in the precipitation with basic lead acetate.

1 drop of concentrated sulfuric acid was added to the 1,240 cc. of filtrate, which was then evaporated to a volume of about 200 cc., interrupting the distillation several times to remove the sediment consisting chiefly of hippuric acid crystals. Diluted sulfuric acid, representing 2 cc. of concentrated sulfuric acid, was added to the 200 cc. of liquid and placed in the ice box overnight to eliminate the large bulk of the hippuric acid which was filtered off. The filtrate was twice extracted with 100 cc. of

TABLE I.

Date.	Weight.	Food ingested.			Urine output in 24 hrs.										
		Protein under investigation.	N	Ben- zoic acid.	Vol- ume.	Total N.		Urea N.		Ammonia N.		Benzoic acid.			Reduc- tion ex- pressed in glucose.
						gm.	cc.	gm.	per cent	gm.	per cent	gm.	per cent	Total.	
1913	kg.		gm.	gm.		gm.		gm.		gm.		gm.		gm.	
Oct. 28	15				230	1.674									
" 29					765	1.469									
" 30					920	0.860	0.223	25.7	0.156	18.0	0.176	0.027	0.149		
" 31		Casein.	6.16		1,460	2.165	1.398	64.6	0.218	10.0					
Nov. 5	15.5	"	6.16		1,045	1.960	1.207	61.6	0.296	15.1	0.189	0.015	0.174		
" 6		"	6.16		1,190	2.611	1.772	67.9	0.374	14.3	0.197	0.004	0.1937		
" 7		"	6.16		1,335	1.831	1.217	66.5	0.163	8.88	0.143	0.010	0.1331		
" 8		"	6.16	16	1,120	4.145	1.718	41.4	0.717	17.3	13.590	0.267	13.323	8.930	
" 9		"	6.16	16	875	4.159	1.493	35.9	0.845	20.3	14.450	0.284	14.166	8.950	
" 10	15.6	"	6.16	16	1,210	4.611	1.856	40.3	0.836	18.1	15.720	0.560	15.160		
" 11		"	6.16		630	2.522	1.576	62.5	0.209	8.3	0.573	0.072	0.501		
" 12	15.6	Casein + gelatin.	12.32	16	1,040	5.635	2.700	47.9	0.815	14.5	13.328	0.115	13.213	2.92	
" 13		" + "	12.32	16	1,100	7.026	3.675	52.3	0.990	14.1	14.326	0.166	14.160	2.94	
" 14		" + "	12.32	24	1,250	9.107	4.929	54.1	1.131	12.4	22.058	0.202	21.856	4.975	

Nov. 15	15.8	Gelatin.	6.16	16	1,230	5.938	2.878	48.5	0.757	12.8	14.810	0.123	14.687	2.407
" 16		"	6.16	16	1,140	6.181	2.756	44.5	0.409	6.6	15.720	0.213	15.507	2.214
" 17		"	6.16	24	1,270	6.584	2.936	44.6	0.729	11.1	21.440	0.535	20.865	4.027
" 18	17	Casein.	6.16		625	3.103	2.122	68.4	0.218	7.0	1.220	0.130	1.09	
" 19		"	12.32	16	1,310	5.414	3.010	55.6	0.586	10.8	13.520	0.280	13.240	6.126
" 20	17.1	"	12.32	16	1,260	8.841	5.498	62.2	1.045	11.8	14.84	0.288	14.552	8.110
" 21		"	12.32	24	1,335	6.838	3.488	51.0	1.117	16.3	17.41*	1.084	16.326	10.96

*On the evening of Nov. 21 the pig showed toxic symptoms. On the following day 4.2 gm. of benzoic acid were eliminated (see p. 568).

TABLE II.

Weight.	Food ingested.			Urine output in 24 hrs.											
	Protein under investigation.	N	Ben- zoic acid.	Total N.	Urea N.		Ammonia N.		Creatinine N.		Benzoic acid.			Reduction expressed in glucose.	
					gm.	per cent	gm.	per cent	gm.	per cent	Total.	Free.	Com- bined.		
kg.		gm.	gm.	cc.	gm.	per cent	gm.	per cent	gm.	per cent	gm.	gm.	gm.	gm.	
19.3	Meat.	6.16	16	1,135	6.832	3.568	52.22	0.647	9.47		14.760	0.238	14.522	6.882	
	"	6.16	16	1,230	4.882	1.981	40.57	0.476	9.75		15.210	0.582	14.628	7.228	
	"	6.16	16	1,120	5.020	1.758	35.0	0.585	11.6		15.47	0.716	14.754	7.643	
	Casein + glycine.	6.16	16	1,280	3.677	0.954	25.95	0.446	12.1	0.274	7.45	14.318	0.248	14.070	2.782
19.7	"	1.87	16	1,360	3.324	0.600	18.06	0.383	11.5	0.287	8.64	15.21	0.400	14.81	2.517
	"	1.87	16	1,700	3.403	0.622	18.30	0.426	12.5	0.287	8.42	17.56	0.722	16.838	2.473
	"	1.87	16	1,260	1.561	0.694	44.5	0.311	19.96	0.256	16.8	0.380	0.017	0.363	
19.9	Casein.	6.16	16	1,060	4.581	1.919	41.9	0.565	12.3	0.264	5.77	15.480	0.315	15.165	6.824
19.8	"	4.19	16	1,340	4.754	1.891	39.8	0.656	13.8	0.314	6.60	15.200	0.616	14.584	7.461
	"	2.09	16	1,200	2.838	0.744	26.2	0.353	12.5	0.236	8.30	14.540	0.556	13.984	7.471
	"		16	1,195	2.736	0.567	20.7	0.278	10.2	0.284	10.6	13.980	1.258	12.722	7.400
				860	1.276	0.247	19.3	0.235	18.6	0.267	20.95	1.281	0.354	0.927	
19.8	Casein.	6.16		560	0.920	0.175	19.0	0.204	22.2	0.265	28.8	0.262	0.056	0.206	

TABLE III.

Weight.	Food ingested.			Urinary output in 24 hrs.											
	Protein under investigation.	N	Benzoic acid.	Vol- ume.	Total N.		Urea N.		Ammonia N.		Creatinine N.		Benzoic acid.		Reduction expressed in glucose.
					gm.	per cent.	gm.	per cent.	gm.	per cent.	gm.	per cent.	Total.	Free.	
kg.		gm.	gm.	cc.	gm.	per cent.	gm.	per cent.	gm.	per cent.	gm.	per cent.	gm.	per cent.	gm.
30.7				1,460	1.486	0.463	31.2	0.218	14.6	0.417	28.1	0.246	0.010	0.236	
30.7	Casein.*	6.16	16	1,435	6.271	2.836	45.2	0.807	12.9	0.486	7.8	15.87	0.308	15.56	7.675
	"	6.16	16	1,640	5.081	2.495	49.1	0.476	9.4	0.537	10.6	15.51	0.560	14.95	6.585
30.9	Beef meat.	7.22	16	1,460	4.052	1.130	27.9	0.654	16.2	0.430	10.6	15.03	0.430	14.60	6.158
	"	7.22	16	1,210	4.258	1.006	23.6	0.819	19.2	0.469	11.0	14.53	0.443	14.087	8.187
30.8	Gelatin.	2.97	16	1,400	3.433	0.612	17.8	0.653	19.9	0.381	11.1	14.76	0.219	14.54	2.871
	"	5.94	16	1,520	4.506	1.223	27.1	0.678	15.0	0.446	9.7	15.74	0.259	15.48	2.580
	"	11.88	16	1,210	9.737	5.420	55.7	0.799	8.2	0.430	4.4	15.24	0.281	14.96	2.314
30.8	Standard starch diet.	None.	0	1,760	5.100	3.422	67.1	0.545	10.7	0.454	8.9	0.777	0.060	0.710	
30.8	"	"	16	1,880	4.297	1.252	29.1	0.614	14.3	0.435	10.1	15.17	0.331	14.84	5.50
	"	"	16	1,330	3.606					0.406	11.3	15.14	1.326	13.81	7.433
	"	"	None.	1,800	2.029	1.147	56.5	0.499	24.6	0.418	20.6	0.567	0.087	0.480	

see two experiments Harris casein, c. p., was used.

toluene. The volume of the liquid was 218 cc. ("A") and 10 cc. of it, diluted to 50 cc., served for the analysis. By reduction it was estimated that "A" contains 25.6 gm. of benzoyl glycuronic acid. There were 12.0 gm. of benzoic acid, which is 1.5 gm. more than 25.6 gm. benzoyl glycuronate contains, showing that hippuric acid was still present in the solution. The solution showed dextro-rotation, and a specific rotation of 27.2° (uncorrected) was estimated for the benzoyl glycuronic acid. The fairly close agreement of the calculated and determined amount of benzoic acid, in which the difference can be attributed to hippuric acid as an impurity, supports the correctness of the proposed quantitative determination of the benzoyl glycuronic acid.

The preparation of benzoyl glycuronic acid in a pure form is postponed for later presentation.

b. *Blood*.—The blood was obtained from the ear vein. The Folin-Wu (40) method for blood sugar was used, and for control we applied the Shaffer-Hartmann method also (41). Hemoglobin determinations were carried out by the procedure of Cohen and Smith (42).

The analytical data are given in Tables I, II, and III.

IV. DISCUSSION OF THE RESULTS.

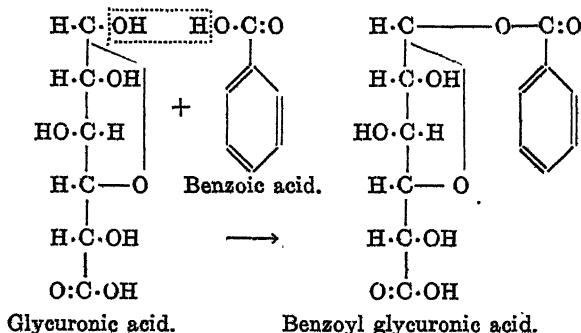
1. *The Relationship of Preformed Glycine to the Glycuronate Formation after Benzoic Acid Ingestion.*

The effect of protein ingestion on hippuric acid formation was shown by Cohn (6) and recently by Griffith and Lewis (9). Both, using rabbits kept under the influence of benzoic acid, investigated the effect of giving them casein and gelatin. Cohn noted an increased output of hippuric acid and the other investigators an increased rate of hippuric acid formation when gelatin was given. Griffith and Lewis collected a 6 hour specimen of urine, which served to establish the rate of hippuric acid elimination. Since 70 per cent of the benzoic acid was eliminated after gelatin ingestion and 45 per cent after casein administration during that period, it is possible that in 24 hour specimens gelatin and casein might not show any difference in hippuric acid formation. The experiments of Cohn, although opposing this hypothesis, are questionable on account of the analytical methods applied.

Judging from Ringer's experiments (15) on a starving goat which had received large quantities of benzoic acid, we expected to find a large amount of free benzoic acid, which we thought would decrease in amount when preformed glycine or a protein containing glycine was given simultaneously.

The analysis of the urine collected on November 8, 9, and 10 (Table I), when casein was the protein under investigation, showed too small an amount of free benzoic acid to serve as an index of hippuric acid formation. The conjugated benzoic acid fraction reached 98 per cent of the total eliminated. The urine was tested with Benedict's (43) qualitative reagent and found to give a moderate reduction. It was then clear that the conjugated benzoic acid did not represent a glycine combination alone but a glycuronic acid compound also. The influence of protein on hippuric acid formation could not be noted solely by a determination of the amount of conjugated benzoic acid, for this remained constant with both casein and gelatin. However, the amount of hippuric acid determined by subtracting the benzoic acid conjugated with the reducing substance from the total combined acid was found to vary. Therefore, the reducing power of the urine rather than the free benzoic acid was used as an accessory factor in the computation of hippuric acid synthesis.

What is the reasoning for the method of calculation of the hippuric acid and what are the analytical findings that support its correctness? That glycuronic acid occurs as the benzoyl ester in sheep's urine after the ingestion of benzoic acid has been convincingly demonstrated by Magnus-Levy (22). We are dealing also with the ester of glycuronic acid and not with an ether form, as shown by the ease of hydrolysis evidenced in its reducing power toward Benedict's qualitative reagent, the alkaline reaction of which is capable of splitting it.



The concentrated urine did not give a typical orcinol test, which shows that we are not dealing with a pentose, but we obtained a positive naphtho-resorcinol test, which is a characteristic reaction for glycuronic acid. The urine showed no gas formation on addition of yeast, and it is known that glycuronic acid is unfermentable. It is commonly stated in text-books that the glycuronic acid does not reduce the Nylander reagent; therefore it is recommended for the differentiation of glycuronic acid from carbohydrates.⁴ This is not true in our case, because the urine tested with this reagent gives a positive test. Therefore, the weight of experimental evidence is fairly conclusive that the reducing material present represents a glycuronic ester of benzoic acid.

An inverse relationship (44) exists between the quantity of the glycuronate eliminated in the urine and the preformed glycine in the food ingested. Compare the experiments of casein ingestion on November 9 and 10 (Table I), in which there was no preformed glycine present in the food with the experiments on November 15 and 16, when approximately 10 gm. of preformed glycine were offered in gelatin. The amount of glycuronic acid present in the urine averaged 9.58 gm. per day, when casein represented the sole protein, compared with 2.49 gm. daily when gelatin was the only source of protein administered. To make it certain that glycine is responsible for the decrease we repeated our casein experiment and gave 10 gm. of glycine in addition, with the result that the elimination of glycuronic acid averaged only 2.77 gm. per day (Table II, December 6, 7, 8). The decrease of the glycuronic acid in the presence of preformed glycine is also manifested when gelatin is given with casein in the diet, being 3.14 gm. per day (Table I, November 12 and 13), a little higher than when gelatin was given alone. If we increase the dose of benzoic acid without changing the other factors we find also an increase in the amount of reducing substance eliminated in the urine (Table I, November 14, 17, and 21).

The question arises: Is the appearance of glycuronate a sign of a temporary exhaustion of glycine, thus preventing hippuric acid synthesis and an additional protective mechanism of the

⁴This test was suggested by Professor S. R. Benedict, who expressed his doubt regarding the correctness of the statement.

body against benzoic acid poisoning? In general, we may say that the body produces more glycuronate in the absence of preformed glycine in the food than when an ample supply of this amino acid is present; therefore, it probably serves as an additional protective mechanism. However, it appears in urine half an hour after benzoic acid ingestion, and it is formed at a greater rate in the early hours than in the later ones. The rate of elimination of the benzoyl glycuronic acid can best be studied when benzoic acid is given alone or together with casein because in these cases larger amounts are excreted and the reducing power of the urine can be conveniently estimated in short intervals.

TABLE IV.
The Rate of Glycuronate Elimination.

Date.	Period.	Urinary output.				Remarks.
		Volume.	Benzoyl glycuronic acid.			
1924		cc.	gm.	gm. per hr.	per cent.	
Feb. 22	10.50 a.m.	169	Slight trace.			At 10.20 a.m.:
	1.05 p.m.	120	0.912	0.405	20.8	Casein.....50 gm.
	4.45 "	134	1.000	0.273	22.9	Benzoic acid. 8 "
" 23	7.45 a.m.	320	2.456		56.3	
" 28	3.45 p.m.		2.247	0.403	60.2	At 10.10 a.m.:
	6.15 "		0.815	0.326	21.8	Boiled rice..200 gm.
" 29	7.45 a.m.		0.672		18.0	Glucose.....50 "
						Benzoic acid..8 "

In the casein experiment 43.7 per cent of the total glycuronate produced was eliminated within 6 hours and 25 minutes; when cooked rice mixed with glucose was given with benzoic acid, essentially a carbohydrate diet, 82 per cent was eliminated in 8 hours and 5 minutes. The continuous elimination of this reducing substance points to a rather slow mobilization of glycine; at least, it shows that a faster absorption of benzoic acid than glycine is offered for the synthesis of hippuric acid. The glycuronic acid, however, offers a substitute detoxicating substance for the free benzoic acid present. That the organism can respond to a greater extent in glycuronate formation than in hippuric acid synthesis, which latter depends upon the available glycine, is

shown in an experiment after giving casein (Table I). When 24 gm. instead of 16 gm. of benzoic acid were ingested there was an increase of glycuronate elimination but no change in the hippuric acid excretion.

What the material is which serves as a precursor for the glycuronic acid produced by the stimulus of the administration of benzoic acid, cannot be definitely stated, although the above experiments point to a protein rather than to a carbohydrate origin.

It has long been known that hippuric acid, when given to an animal, is eliminated quantitatively in the urine (12, 13). The view has been propounded that this substance undergoes hydrolysis to its components by a specific enzyme and is then resynthesized into hippuric acid. We administered to the pig 10 gm. of hippuric acid dissolved in 150 cc. of water and neutralized with sodium hydroxide and found after 8 hours and 15 minutes 6.26 gm. of hippuric acid eliminated; in the 24 hour specimen we recovered 10.11 gm. of hippuric acid, a little more than that ingested because the pig's urine contains normally some hippuric acid. We repeated this experiment several times, but in no case did the urine reduce Benedict's qualitative reagent. This observation does not support the theory that hippuric acid is split before absorption, nor does the fact that when glycine in a larger amount than the molecular equivalent was given simultaneously with benzoic acid some glycuronate always appeared in the urine.

2. Does Benzoic Acid Cause an Increased Protein Catabolism?

McCollum (45), in his studies on the utilization of different proteins in pigs, showed that casein was sufficient for maintenance and growth when it was the only source of protein; while gelatin diminished the "wear-and-tear" quota of the protein metabolism by only 40 per cent; and even when it was fed in great excess over the maintenance needs of the pig there was no evidence obtained with regard to the formation from it of new body tissue.

In the experiments here reported a preliminary period of pure starch ingestion showed a minimum urinary nitrogen, representing the "wear-and-tear" quota of 0.868 gm. on October 30, 1923 (Table I), or 56 mg. of nitrogen per kilo of body weight. On the following 3 days casein was given with the standard diet in an amount several times greater than the "wear-and-tear"

quota and two-thirds of it was deposited. This remarkable storage of protein nitrogen fell one-half when benzoic acid was added to the daily food. The total excretion of urinary nitrogen for the 3 day periods after giving casein alone was 6.40 gm., and in the case of casein plus benzoic acid was 12.92 gm. The intake in both cases was 18.48 gm. of nitrogen for each period. On the other hand, when 18.48 gm. of gelatin nitrogen was the only protein nitrogen taken with benzoic acid, on November 15, 16, and 17, 1923 (Table I), the urine showed 18.70 gm. of nitrogen eliminated, which represents a small negative balance. When the same amount of meat nitrogen was consumed with benzoic acid, only 1.75 gm. of nitrogen were retained.

These experiments show an increased protein catabolism when benzoic acid is administered in large quantities to the pig, which

TABLE V.

(In periods of 3 days.)

Date.	Diet.	N intake.	N in urine.
		gm.	gm.
Nov. 5, 6, 7	Standard + casein.	18.48	6.4
" 8, 9, 10	" + " + benzoic acid.	18.48	12.92
" 15, 16, 17	" + gelatin + " "	18.48	18.70
Dec. 3, 4, 5	" + meat + " "	18.48	16.73

expresses itself in a reduced ability to store protein. The pig normally deposited 64 per cent of the casein nitrogen (the fecal nitrogen being neglected), but when benzoic acid was added it stored only 30 per cent of it. In this sense we may state that the benzoic acid ingestion tends to elevate the protein metabolism. This may be due to the continual withdrawal of glycine, a necessary constituent for the synthesis of body protein. We did not aim to establish nitrogen equilibrium and to study the effect of various proteins on the hippuric acid production at that level, for with gelatin alone nitrogen equilibrium is impossible, and with the other proteins the amounts given would have been too small to serve our purpose. However, we instituted a series of experiments with a decreasing quantity of protein nitrogen intake (Table

II, December 10, 11, and 12, 1923) followed by a day on which the pig received the standard starch diet and benzoic acid, and the next day the same food without the benzoic acid. The urinary nitrogen, when contrasted with the nitrogen intake, showed a negative balance except on the 1st day (December 10), but if we compare that day with December 15, when the diet was identical except that no benzoic acid was administered, the elevation in protein metabolism is unquestionable. Furthermore, the nitrogen in the urine is more than double that on December 13, when benzoic acid was added to the standard starch diet, if compared to the nitrogen elimination on the following day without benzoic acid.

The creatinine elimination, as shown in Tables II and III, was practically constant. Since the diet was free of creatinine, one may conclude that the endogenous protein metabolism is affected by neither protein ingestion (casein and gelatin) nor the administration of benzoic acid. A constant creatinine output, however, does not exclude an increased protein metabolism. We may quote two instances in which creatinine elimination is constant, or even lowered, although a pronounced elevation of protein metabolism exists. First: in a normal starving animal or in a starving man, there is a high urinary nitrogen output lasting for a few days, and concomitant with this the creatinine eliminated is constant or even lowered. In this case the increased protein metabolism is attributed to the catabolism of deposit protein. Secondly, the creatinine output is unchanged in phlorhizinized dogs during starvation and also when muscle protein is fed to them, as shown by S. R. Benedict (46). Although the protein metabolism is several times higher with phlorhizinized dogs, the creatinine excretion is unchanged from that observed in the same starving animal previous to phlorhization. Therefore, the constant creatinine output before and during benzoic acid ingestion cannot be used as evidence against an increased protein metabolism.

3. Experiments on Giving Protein Simultaneously with Benzoic Acid.

If glycine synthesis plays an all-important rôle in hippuric acid formation when benzoic acid is administered, then we should

find no difference in the hippuric acid elimination when the protein supplied contains preformed glycine. The toxic effect noted after large quantities of benzoic acid are given should occur always when a certain dosage is administered; that is, after the rate of glycine synthesis has reached its maximum and the amount produced is insufficient to counteract the toxic effect of benzoic acid. We may state in advance that our results do not confirm these theoretical arguments.

When 16 gm. of benzoic acid are given concomitantly with 41.5 gm. of gelatin, which contains approximately 10 gm. of glycine, or a sufficient amount to cover the glycine requirement for hippuric acid formation, 89.6 per cent of the combined benzoic acid eliminated appears as hippuric acid, while if gelatin is replaced by an equivalent amount of casein the hippuric acid part represents only 68 per cent. We may even double the amount of casein, yet there is no increase in the hippuric acid formation suggesting that an increased protein metabolism does not necessarily influence the magnitude of glycine synthesis. In the experiments of November 10 and 19, when 6.16 and 12.32 gm. of nitrogen in casein with the same amount of benzoic acid were administered, 13.35 and 13.34 gm. of hippuric acid were excreted. That the limit of glycine synthesis was actually reached is shown in the experiment of November 21, 1923 (Table VI) in which we increased the amount of benzoic acid from 16 to 24 gm., the pig receiving 12.32 gm. of casein nitrogen daily. The amount of hippuric acid eliminated was the same as before or 13.07 gm. This represents, therefore, the largest quantity of glycine which the pig was capable of synthesizing during the early period of experimentation. On the other hand, with a gradual decrease in the amount of casein ingested (Table VIII), the amount of hippuric acid eliminated shows a slight but not proportional decrease, and one might justly conclude from this series that when a protein which is free from preformed glycine is ingested in small quantities it does provide conditions for a slight increase in hippuric acid formation. But we may add that, when a limit of glycine synthesis is reached, the administration of an additional quantity of a glycine-free protein, like casein, is without effect on glycine production.

On the evening of November 21, 1923, the pig showed toxic symptoms, such as vomiting and nervousness. The 24 hour urine of this day indicated that 6.6 gm. of benzoic acid had been retained in the body of the animal. The following day the pig refused both food and water and blindness developed toward evening. Therefore, 10 gm. of gelatin in 500 cc. of milk were administered at 5 p.m. The pig shortly regained health and appetite and 2 days later the eyesight was restored (Table I).

With gelatin, the pig tolerated 24 gm. of benzoic acid without showing any signs of toxicity or discomfort. This clearly demonstrates that preformed glycine was a factor, for the animal was able to produce twice the amount of hippuric acid that was formed after casein ingestion. The failure to detoxicate the excess of benzoic acid with glycine in the latter experiment must be attributed to the insufficiency of the function for the synthesis of glycine rather than to an inability to perform the chemical union of benzoic acid with glycine.

The toxic effect seems to be due to the slow elimination and consequent retention of the ingested benzoic acid; however, when sufficient preformed glycine is present in the food, as is the case when gelatin is fed, the rate of elimination is increased and thus the deleterious effect is avoided—a procedure which may be used for therapeutic treatment to counteract benzoic acid poisoning.

Parker and Lusk (5) measured the synthetic formation of glycine from the relation of total nitrogen to hippuric acid nitrogen; i.e., glycine N: nitrogen ratio. A ratio greater than 4.7, which exists when the glycine in the body protein is utilized in the formation of hippuric acid, indicates a synthetic production of glycine. Naturally, this ratio is valid only in starvation; when a glycine-free diet is taken its magnitude depends upon the character of nitrogen balance.

On December 13, 1923, when the pig was given the standard starch diet (nitrogen-free), the ratio was 32.3 (Table VIII). On the previous day the ratio had been 36, and this may be considered the highest ratio found in our work, fulfilling the restrictions pointed out above. The influence of the total nitrogen on the magnitude of the ratio is well demonstrated by comparing the experiment of November 13, when casein and gelatin were admin-

istered, with that of December 7 in which the gelatin was substituted by glycine equivalent to the amount present in gelatin. The amounts of hippuric acid nitrogen eliminated in the two experi-

TABLE VI.
Varieties of Protein as Factors in Hippuric Acid Production.

Date.	In diet.			Benzoic acid given.	Urinary output.			Urinary acid N.	
	Protein.	Total N.	Glycine N.		Hippuric acid.	Hippuric acid N.	Total N.	Hippuric acid N.	Total N.
<i>1923-24</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	
Nov. 8	Casein.	6.16	0	16	10.67	0.834	4.145	20.1	
" 9	"	6.16	0	16	11.90	0.931	4.159	22.4	
" 10	"	6.16	0	16	13.35	1.044	4.611	22.7	
" 12	Casein + gelatin.	12.32	1.94	16	16.48	1.289	5.635	22.8	
" 13	" "	12.32	1.94	16	17.86	1.397	7.026	19.9	
" 14	" "	12.32	1.94	24	27.12	2.120	9.107	23.3	
" 15	Gelatin.	6.16	1.94	16	19.16	1.498	5.938	25.2	
" 16	"	6.16	1.94	16	20.55	1.607	6.181	26.0	
" 17	"	6.16	1.94	24	26.60	2.080	6.584	31.6	
" 19	Casein.	12.32	0	16	13.34	1.043	5.414	19.3	
" 20	"	12.32	0	16	13.29	1.039	5.841	11.8	
" 21	"	12.32	0	24	13.07	1.022	6.838	15.0	
Dec. 3	Beef meat powder.	6.16	0.26	16	14.46	1.131	6.832	16.6	
" 4	" " "	6.16	0.26	16	14.27	1.116	4.882	22.9	
" 5	" " "	6.16	0.26	16	14.05	1.099	5.020	22.0	
" 6	Casein + glycine.	8.03	1.87	16	15.28	1.194	3.677	32.5	
" 7	" "	8.03	1.87	16	16.87	1.319	3.324	39.7	
" 8	" "	8.03	1.87	16	19.93	1.558	3.403	45.8	
" 10	Casein.	6.16	0	16	15.46	1.209	4.581	26.4	
Mar. 25	"	6.16	0	16	15.20	1.188	6.271	18.9	
" 26	"	6.16	0	16	15.38	1.203	5.081	23.7	

ments were 1.397 and 1.319 gm., respectively, yet the ratios were 19.9 and 39.7, although there was a larger amount of hippuric acid formation in the first case (Table VI).

TABLE VII.
Showing the Relationship in Percentage of Benzoic Acid Partition.

Food ingested.			Benzoic acid eliminated in the urine.									
Protein under investigation.	N	Benzoic acid.	Total.		Combined.				With glycuronic acid.		Free.	
			gm.	per cent of intake	gm.	per cent of total	With glycine.		gm.	per cent of combined	gm.	per cent of total
							gm.	per cent of combined				
Casein.	6.16	16	13.59	84.9	13.32	98.0	7.27	54.6	6.05	45.4	0.267	2.0
"	6.16	16	14.45	90.3	14.17	98.1	8.12	57.3	6.05	42.7	0.284	1.9
"	6.16	16	15.72	98.3	15.16	96.4	9.10	60.0	6.06	40.0	0.560	3.6
Casein + gelatin.	12.32	16	13.33	83.3	13.21	99.1	11.23	85.0	1.98	15.0	0.115	0.9
" + "	12.32	16	14.33	89.6	14.16	98.8	12.17	86.0	1.99	14.0	0.166	1.2
" + "	12.32	24	22.06	91.9	21.86	99.1	18.49	84.6	3.37	15.4	0.202	0.9
Gelatin.	6.16	16	14.81	92.6	14.69	99.2	13.06	88.9	1.63	11.1	0.123	0.8
"	6.16	16	15.72	98.3	15.51	98.7	14.01	90.3	1.50	9.7	0.213	1.3
"	6.16	24	21.44	89.3	20.86	97.3	18.13	86.9	2.73	13.1	0.585	2.7
Casein.	12.32	16	13.52	84.5	13.24	97.9	9.09	68.7	4.15	31.3	0.280	2.1
"	12.32	16	14.84	92.8	14.55	98.1	9.06	62.3	5.49	37.7	0.288	1.9
"	12.32	24	17.41	72.5	16.33	93.8	8.90	54.6	7.43	45.4	1.084	6.2
Beef powder.	6.16	16	14.76	92.3	14.52	98.4	9.85	67.8	4.67	32.2	0.298	1.6
"	6.16	16	15.21	95.1	14.63	96.2	9.73	66.6	4.90	33.5	0.582	3.8
"	6.16	16	15.47	96.7	14.75	95.4	9.57	64.9	5.18	35.1	0.716	4.6

Casein + glycine.	8.03	16	14.32	89.5	14.07	98.3	10.41	74.0	3.66	26.0	0.248	1.7
" + "	8.03	16	15.21	95.1	14.81	97.4	11.50	77.7	3.31	22.3	0.400	2.6
" + "	8.03	16	17.56	109.7	16.84	95.9	13.59	80.7	3.25	19.3	0.722	4.1
Casein.	6.16	16	15.48	96.8	15.17	98.0	10.54	69.5	4.63	30.5	0.315	2.0
"	6.16	16	15.87	99.2	15.56	98.1	10.35	66.5	5.21	33.5	0.308	1.9
"	6.16	16	15.51	96.9	14.95	96.4	10.48	70.1	4.47	29.9	0.560	3.6
Beef meat.	14.44	32	29.56	92.4	28.69	97.1	18.96	66.1	9.73	33.9	0.873	2.9
Gelatin.	5.94	16	15.74	98.4	15.48	98.4	13.73	88.7	1.75	11.3	0.259	1.6
None.		16	13.98	87.4	12.72	91.0	7.70	60.5	5.02	39.5	1.258	9.0
"		16	15.17	94.8	14.84	97.8	11.11	74.9	3.73	25.1	0.331	2.2
"		16	15.14	96.3	13.81	99.7	8.77	63.5	5.04	36.5	1.326	10.3

This example leads us to a very instructive fact regarding the nitrogen elimination in the casein and glycine series of experiments (Table II). The glycine administered contained 1.87 gm. of nitrogen, of which 1.558 gm. reappeared in the urine on the 3rd consecutive day of this series as hippuric acid nitrogen. One may safely suppose that the rest of the glycine nitrogen appeared in the urine as urea nitrogen, which would leave 1.53 gm. of nitrogen attributable to the protein metabolism. On the following day the same quantity of casein, with the same standard starch diet, was ingested without benzoic acid. The total nitrogen eliminated was 1.56 gm., showing that if a sufficient amount of preformed glycine is present in the food to combine with the benzoic acid given, the protein metabolism is not affected, and the casein may be normally retained and deposited in a growing pig. Benzoic acid in the presence of glycine becomes harmless.

It may be mentioned here that this conclusion was confirmed by unpublished respiration calorimeter experiments, which the author has carried out in collaboration with Drs. D. Rapport and R. Weiss (see following paper).

The hippuric acid formation is in proportion to the glycine content to the protein ingested—it is highest when gelatin is given, less with meat protein, and the least with casein. In the last case the glycine production is a result of synthesis, since casein is free from glycine as far as we know at present.

The urea elimination after benzoic acid ingestion has been a subject of considerable argument. A superficial examination of Tables I and II indicates that the urea excretion drops in proportion to the increase in percentage of hippuric acid. The sum of urea, ammonia, and hippuric acid nitrogen generally represents the amount of urea nitrogen found on those days when benzoic acid was omitted from the diet.

Glycine, which is normally converted into urea, escapes this conversion by combining with benzoic acid, therefore the percentage of urea nitrogen must diminish. Urea nitrogen itself cannot serve as a precursor for glycine synthesis because urea administered alone with benzoic acid does not influence the hippuric acid output.

Table VII summarizes the data concerning the elimination of benzoic acid. The most outstanding feature is the uniformity of

the combined form. We may safely state that 97 to 99 per cent of the eliminated benzoic acid is present in the conjugated form and the rest is eliminated as free acid. The constancy in the percentage of the conjugated form is misleading if one does not consider the two compounds which appear in union with the benzoic acid; *i.e.*, glycine and glycuronic acid. The latter was discussed in a previous chapter, so we will deal at present only with glycine as a factor of conjugation. Excepting 1 day when toxic symptoms were present we find the lowest percentage of benzoic acid combined with glycine to be 60 per cent. On this day no protein food

TABLE VIII.

The Variation in the Quantity of Protein as a Factor in Hippuric Acid Production When 16 Gm. of Benzoic Acid Are Given Daily.

Date.	In diet.			Urinary output.				
	Protein.	Total N.	Glycine N.	Glycuronic acid.	Benzoic acid combined.		Hippuric acid.	Hippuric acid N.
					With glycuronic acid.	With glycine.		
1923-24		gm.	gm.	gm.	gm.	gm.	gm.	gm.
Nov. 19	Casein.	12.32	0	6.60	4.15	9.09	13.34	1.043
Dec. 10	"	6.16	0	7.35	4.63	10.54	15.46	1.209
" 11	"	4.19	0	8.04	5.06	9.52	13.97	1.093
" 12	"	2.09	0	8.05	5.07	8.92	13.08	1.023
" 13	None.		0	7.98	5.02	7.70	11.30	0.884
Mar. 31	Gelatin.	2.97	0.93	3.09	1.95	12.59	18.47	1.445
Apr. 1	"	5.94	1.87	2.78	1.75	13.73	20.15	1.540
" 2	"	11.88	3.74	2.49	1.57	13.39	19.65	1.536
" 4	None.		0	5.50	3.73	11.11	16.30	1.274
" 5	"			8.01	5.04	8.77	12.87	1.006

was given and the hippuric acid nitrogen to total nitrogen ratio was 32.3, indicating a synthetic production of glycine. When casein was added to the diet the part combined with glycine reached 68 per cent, and since the origin of glycine is also conceded to be the result of synthesis, we may justly conclude that the synthesis was slightly and favorably influenced by the casein intake.

In the experiments with gelatin (Table VIII) the amount of this protein was increased on successive days in order to study the quantitative relation between its glycine content and the synthetic

formation of hippuric acid in the animal body. The lowest intake of one-half molecular equivalent of glycine to one of benzoic acid produced only slightly less hippuric acid than one in which four times as much glycine was offered (two molecular equivalents).

This confirms the fact that a limited amount of synthetic glycine is always at the disposal of the organism. This interpretation explains the experimental findings in Table VIII.

An increase in the dosage of benzoic acid from 16 gm. daily to 24 gm. shows an increased hippuric acid production when gelatin or gelatin plus casein are given, but not when casein alone is administered. In the first two experiments there was not enough preformed glycine present to cover the theoretical combining power of benzoic acid. Approximately 10 per cent of the ingested benzoic acid was not recovered in the urine and about 85 per cent of the conjugated form of benzoic acid was eliminated in the form of hippuric acid. Although the absolute amount of hippuric acid produced was larger when the dosage of benzoic acid was raised from 16 to 24 gm., there was no material change in the percentage of benzoic acid combined with glycine; *i.e.*, the manner of hippuric acid synthesis or of glycine production was not appreciably influenced by this change in dosage. In both cases the same percentage of benzoic acid conjugated with glycuronic acid appeared in the urine (Tables VI and VII).

The protein metabolism *in vivo* produces glycine in the same amount as that obtained on acid hydrolysis *in vitro*. The hypothesis that there is a synthetic production of glycine is well founded and is clearly demonstrated in the casein experiments, especially in those in which no protein was administered with the benzoic acid, if we examine the ratio of hippuric acid nitrogen and total urinary nitrogen, as suggested by Parker and Lusk. However, this glycine synthesis is of a limited extent and may be estimated by determining the lethal dose of benzoic acid.

The magnitude of the specific dynamic action of various proteins cannot be interpreted solely as due to one specific amino acid though that amino acid is a high heat producer when it is ingested and metabolized alone, *i.e.* glycine, because then we would also find a relationship of preformed glycine content to the extra heat produced after protein ingestion. The experiments with casein, meat, and gelatin in relation to the hippuric acid formation

reported in this paper do not support the theory of unlimited glycine synthesis from proteins which lack this amino acid. Proteins in general liberate glycine in quantities similar to the amounts found *in vitro* after acid hydrolysis.

4. *The Rate of Benzoic Acid Elimination.*

In this part of the investigation some changes in the diet were instituted in order to be able to observe the effect of the proteins alone on the rate of benzoic acid elimination. The 300 gm. of starch were given in the afternoon at 5 o'clock, mixed with 1 liter of milk. The amount of benzoic acid was the same as in the previous experiments recorded in Tables I, II, and III; *i.e.*, 16 gm. daily. The experiments in Table IX represent only the rate of elimination of 8 gm. of benzoic acid, which were given in the morning with the material indicated in the second column; the remaining 8 gm. were administered in the afternoon. The purpose of giving the second dose in the afternoon was to keep the animal constantly under the influence of benzoic acid. Although the morning urine was not always timed exactly, the experiments carried out previously showed that the benzoic acid which was given at 5 o'clock in the afternoon was all eliminated by the morning; therefore, it was immaterial whether or not the bladder was emptied at the beginning of the period. The pig was watched and the time of the first urination was noted. During the interval the pig was in the respiration calorimeter he passed no urine, but he usually did so shortly after he was returned to his cage. In Column 3 the duration of the period which elapsed from the administration of the benzoic acid until urination is given.

The elimination of benzoic acid occurs in three forms: (1) free, (2) combined with glycine, and (3) combined with glucuronic acid. The combined forms are the ones of chief interest, since the changes in the free benzoic acid are slight and irregular. The rate of hippuric acid elimination depends directly upon the quantity of available glycine (preformed) and is highest in those experiments in which this requirement is amply covered. It is lower when casein is fed to the pig and lowest when benzoic acid is given alone.

When benzoic acid is given with casein about 27 per cent of the conjugated benzoic acid eliminated is combined with glycuronic acid; when casein is omitted, 25 per cent of the conjugated benzoic acid occurs as a glycuronate ester.

The inverse proportion of preformed glycine is clearly seen from the above experiments. The rate of hippuric acid formation shows also the limitation of glycine synthesis; when we compare the casein experiment with that of casein plus glycine, we find an increase of hippuric acid formation in the latter instance. Although the benzoic acid absorption was probably the same whether or not the glycine was administered, the rate of its elimination was slightly increased in the presence of this amino acid. Therefore, a decreased absorption of benzoic acid is not responsible for the lowered hippuric acid production in the casein experiment, but a deficiency in glycine synthesis. Thus, when the glycine synthesis has reached its maximum, the excess of benzoic acid is combined with glycuronic acid. This view-point is in harmony with the result observed by Griffith and Lewis (47) with regard to benzoic acid absorption, since these investigators found that the extent of elimination of benzoic acid was independent of the path of administration, but was identical when given orally or by intravenous injection.

5. Changes in Blood Sugar during Benzoic Acid Ingestion.

It was of interest to find out whether or not the blood sugar level of the animal undergoes any change on account of the appearance of the glycuronate in the urine as a result of benzoic acid ingestion. The glycuronic acid being a reducing agent, one might consider that it might influence the blood sugar figures, which represent in reality a sum of all reducing substances present in the blood; therefore, it would naturally increase temporarily the blood sugar level. An increase might indicate also a carbohydrate mobilization which might be related to the production of glycuronic acid.

In the experiments reported here 8 gm. of benzoic acid were administered by a stomach tube and casein (6.16 gm. of nitrogen), suspended in water, was eaten by the pig voluntarily. The results are given in Table X.

The blood sugar falls instead of showing an expected elevation in consequence of a high rate of glycuronate formation and elimination during the early hours after benzoic acid ingestion. However, the hemoglobin percentage offers a possible explanation because the decreased hemoglobin content noted indicates a dilution process, which may be caused by the entrance of the hippuric acid and glycuronic ester into the blood stream.

TABLE X.

The Effect of Benzoic Acid on the Reducing Property of the Blood.

Time.	Hemoglobin	Blood sugar.		Remarks.
		Folin-Wu.	Shaffer-Hartmann.	
	per cent	per cent	per cent	
10.20 a.m.		0.080		Jan. 19, 1924, fasting.
11.25 "		0.065		At 10.22 a.m. 8 gm. benzoic acid +
12.25 p.m.		0.084		casein (6.16 gm. N) + 300 cc.
1.25 "		0.077		water.
3.25 "		0.074		
10.05 a.m.	124.0	0.086	0.079	Jan. 26, 1924, fasting.
11.15 "	112.7	0.075	0.061	At 10.15 a.m. 8 gm. benzoic acid +
12.15 p.m.	110.0	0.075	0.070	casein (6.16 gm. N) + 300 cc.
1.15 "	103.4	0.079	0.067	water.
3.15 "	119.0	0.075	0.061	
10.07 a.m.	129.3	0.084	0.065	Feb. 2, 1924, fasting.
11.15 "	136.3	0.111	0.087	At 10.10 a.m. 300 gm. starch (corn)
12.15 p.m.	98.0	0.108	0.092	+ 300 cc. water.
1.15 "	102.7	0.073	0.065	
2.15 "	98.0	0.110		
3.15 "	102.7	0.111		

To demonstrate a real case of carbohydrate mobilization we fed the pig with corn-starch and so produced a transitory hyperglycemia and, judging from the hemoglobin figures, a pronounced dilution of the blood. Fisher and Wishart (48) observed similar changes in the blood of dogs after the ingestion of large amounts of glucose. The highest blood sugar figures were found in the 1st

hour after the ingestion of corn-starch, whereas in the experiments on casein plus benzoic acid the lowest figure was obtained in the 1st hour. These findings do not support the idea that benzoic acid appreciably influences the blood sugar level. The excretion of the glycuronate from the body must be very rapid, since it does not increase the reducing power of the blood; on the other hand, if its production takes place in the kidney an immediate elimination would make such a study useless.

V. SUMMARY AND CONCLUSIONS.

1. Benzoic acid fed to a pig is eliminated in the urine as free acid and in conjugation with glycine (hippuric acid) and in conjugation with glycuronic acid (benzoyl glycuronic acid).

2. When starch is the only food supply, the ingestion of 16 gm. of benzoic acid stimulates hippuric acid formation, and 60 per cent of the combined benzoic acid represents glycine conjugation.

3. Casein, which does not contain glycine, given simultaneously with benzoic acid, increases the hippuric acid production slightly above that formed on a starch diet with benzoic acid administration. The percentage of benzoic acid combined with glycine reaches 68. The glycine used in hippuric acid formation, when no preformed glycine is offered in the food, originates partly from catabolized body protein and partly from synthesis. The quantity of glycine available from both sources mentioned is limited, and after its maximum has been reached there is no difference in hippuric acid formation either by increasing the casein nitrogen in the diet from 6.16 to 12.32 gm. or by increasing the dose of benzoic acid from 16 to 24 gm. The excess benzoic acid is retained or only slowly eliminated and thus results in benzoic acid intoxication.

4. When glycine as such, or preformed glycine in the shape of gelatin, is added to a casein diet in a quantity representing approximately one molecular equivalent, the amount of hippuric acid eliminated in both experiments is the same. The percentage of benzoic acid combined with glycine reaches 85, while gelatin alone gives the even higher value of 89 per cent.

5. If an amount of glycine which is more than sufficient to combine with the benzoic acid is administered with casein as the only protein in the diet, the protein metabolism is not affected and

casein may be normally retained and deposited in a growing pig. If glycine is not added to this diet, benzoic acid causes an increased metabolism of protein. Benzoic acid in the presence of glycine becomes harmless. Gelatin which contains glycine also detoxicates benzoic acid to the extent of its glycine content.

6. Meat protein in an amount used in this investigation contains too small an amount of glycine to show a pronounced effect on the hippuric acid production. However, the benzoic acid conjugated with glycine represents 66 per cent of the combined, which is a higher level than the average of all the experiments when casein was given (63 per cent), 16 gm. of benzoic acid having been administered in this series.

7. The rate of hippuric acid elimination depends directly upon the quantity of available (preformed) glycine and is highest in the case of gelatin; it is lower when casein is fed, and lowest when benzoic acid is given alone.

8. When benzoic acid is administered in large quantities to a pig this animal eliminates benzoyl glycuronic acid. Other investigators working with pigs as subjects for experimentation failed to observe this fact. A method of determination of this compound is presented, based on its reducing property, and thereby a more reliable technique for the hippuric acid determination has been obtained.

9. The amount of glycuronic acid averaged 9.58 gm. a day, when casein represented the sole protein, compared with 2.49 gm. daily when gelatin was the only source of protein administered. 10 gm. of glycine added to the casein diet resulted in a daily elimination of 2.77 gm. of glycuronic acid, showing that the glycine in the gelatin is responsible for the decrease in glycuronate formation.

10. The presence of this reducing substance in the urine did not increase the blood sugar level in the first 5 hours when casein was given simultaneously with benzoic acid, although approximately 40 per cent of the benzoyl glycuronic acid present in a 24 hour urinary specimen was found to be eliminated in the first 6 hours after giving benzoic acid. After a starch diet 80 per cent of the total may appear in the urine within 8 hours.

11. In a limited sense an inverse relationship exists between the glycine content of the protein fed and the quantity of gly-

curonate eliminated after benzoic acid is administered and a direct proportion between the amount of this amino acid and the hippuric acid excreted.

12. Since the specific dynamic actions of equal amounts of meat, gelatin, and casein are practically identical, it is evident that this cannot depend upon an equality of glycine production after their several administrations to an animal.

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ANIMAL CALORIMETRY.

TWENTY-EIGHTH PAPER.

THE RESPIRATORY METABOLISM OF A YOUNG PIG AS INFLUENCED BY FOOD AND BENZOIC ACID.

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I. INTRODUCTION.

The preceding paper by Csonka led to the inquiry whether, after giving benzoic acid to a pig, there would be a reduction in the heat production of the animal as a consequence of the withdrawal of glycine and its stimulating effect upon the metabolism. Some of the work accomplished on the dog and reported in "Animal calorimetry, Paper XXVI" was repeated with the pig and incidentally the production of fat from carbohydrate was investigated.

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II. THE BASAL METABOLISM.

A young Berkshire hog 8 weeks old (on the testimony of the farmer who bred him) and weighing 16.7 kilos was received into the laboratory on October 25, 1923. His measured length from nose to buttocks and his weight were thus recorded:

Date.	Age.	Length.	Weight.
1923-24	days	cm.	kg.
Oct. 25	56	60	16.7
Dec. 17	78		20.0
" 26	88	90	20.2
Jan. 28	121	94	27.2
Apr. 2	186		30.7
May 8	222	107	

Several determinations of the basal metabolism were made between the 78th and 186th days of the life of this young animal. The "standard diet" contained 300 gm. of corn-starch and 1 liter of milk (about 5.5 gm. of N). Since the pig, when partaking of this diet, eliminated 1.6 gm. of urinary nitrogen on December 27 and 2.8 gm. on January 23, it is evident that there was very little protein available for normal growth. Other factors limiting growth were the influence of frequent administrations of benzoic acid, a reduction in the caloric intake when starch was at times omitted from the evening meal and milk alone was given. On the other hand, the occasional addition of casein to the diet of the animal favored the growth process.

Under ordinary circumstances, Joseph (1) shows that a pig may increase in weight from 23 to 113 kilos in 174 days and he also comes to the significant conclusion: "When the supply of protein is deficient either quantitatively or qualitatively it seems that only the *amount* of the body protein is affected while the *character* of the proteins formed in the various tissues is unchanged."

Deighton's hog (2), which led a normal, active life and was excellently nourished, grew from a weight of 12.8 kilos at an age of 75 days to one of 88 kilos at an age of 184 days. It is evident that limitation of the protein factor in the diet, together with restricted confinement in a small cage over a long period of

time, prevented the normal growth of the pig in this laboratory and may partly explain the curious results presented in Table I.

In work with the pig very high respiratory quotients and an increased metabolism were observed even 18 hours after giving 300 gm. of starch. For this reason only 1 liter of milk was given on those days when the true basal metabolism was to be determined on the morning of the following day. This differentiation between the basal metabolism during periods of very high and the usual respiratory quotients was made only after the experiments had been carried on for some time and hence this factor pervades the research.

It is quite striking that the basal metabolism of the pig scarcely changed from December 17, when it weighed 20 kilos, and was 78 days old, until April 2, when the weight was 30.7 kilos and the age 186 days. The average figure is 28.6 calories per hour. If one excepts Experiment 41, during which the pig was probably sound asleep, the other experiments show an extreme variation of ± 3 per cent.

The quantity of heat lost by vaporization of water averaged exactly 20 per cent of the total calories, a result similar to that obtained on man and on the dog.

When *high respiratory quotients* were present the heat production showed an average increase of 24 per cent above the basal level. This high metabolism betokens a plethora of glucose molecules available for oxidation and the high quotients a conversion of carbohydrate into fat, a question which will be dealt with later.

Deighton states that his growing pig manifested a metabolism of 1,032 calories per square meter of surface daily, whereas the older animal of Capstick and Wood (3) eliminated 906 calories. The formula

$$\text{Surface} = 9 \sqrt[3]{\text{Weight}}$$

was used. A similar calculation for our animal reveals the following relations.

	1923	1924
Date.....	Dec. 17	Apr. 2
Weight, kg.....	20	30.7
Area, sq. m.....	0.663	0.882
Calories in 24 hours (average of the period)....	686	686
“ per sq. m. surface.....	1,035	778

TABLE I.
The Basal Metabolism.

Food on day before.	Experiment No.	Date.	R.Q.	CO ₂	O ₂	Urinary N.	Calories.		Weight.	Age.
							Indirect.	Direct.		
		1923-24		gm.	gm.	gm.			kg.	days
Milk, 1 liter, corn-starch, 300 gm., benzoic acid, 8 gm.	1	Dec. 17	0.91	10.26	8.21	0.004	28.24	35.08	20.0	78
The same diet.	2	" 18	1.03	11.40	8.04	0.073	28.37	29.58	20.2	79
" " "	7	" 27	0.93	10.64	8.29	0.055	28.75	28.76	22.0	89
" " "	16	Jan. 8	0.96	11.29	8.55	0.073	29.79	31.55	24.2	101
Milk, 1 liter.	27	" 24	0.81	9.86	8.84	0.073	29.65	29.87	26.0	117
" 1 "	33	Feb. 5	0.78	9.08	8.42	0.110	27.96	27.35	27.7	129
" 1 "	41	" 16	0.77	8.53	8.01	0.267	26.22	26.46	27.7	140
" 1 "	45	" 25	0.77	9.37	8.86	0.094	29.35	24.52	28.6	149
" 1 "	50	Apr. 2	0.78	9.58	8.98	0.143	29.11	29.04	30.7	186
Average.			0.86	10.00	8.47	0.106	28.90	29.13		
Milk, 1 liter, corn-starch, 300 gm., and hippuric acid, 10 gm.	26	Jan. 23	1.18	16.43	10.09	0.073	36.52	36.63	26.6	
Milk, 1 liter, corn-starch, 300 gm., and corn-starch and glucose.	31	Feb. 1	1.22	16.46	9.82	0.058	35.66	39.08	27.8	
Milk, 1 liter, corn-starch, 300 gm., and rice and glucose.	44	" 21	1.22	15.90	9.50	0.068	34.63	31.38	29.0	
Milk, 1 liter, corn-starch, 300 gm., and rice and benzoic acid.	48	" 29	1.17	15.71	9.78	0.068	35.30	31.66	29.9	
Average.			1.20	16.12	9.80	0.116	35.53	34.69		
							24.3 per cent increase over the basal (28.6 calories).			

A fall in the basal metabolism per square meter of surface is a usual accompaniment of protein undernutrition (4). The maintenance of a constant level of basal metabolism in a stunted young animal has not heretofore been observed. It finds its only counterpart in the fact that boys of thirteen produce the same number of calories of basal metabolism as their fathers, as found by Du Bois (5), and girls of both twelve and seventeen have an average basal heat production of 1,250 calories per day irrespective of age, as found by Benedict and Hendry (6). These facts, however, have to do with the age of the protoplasm. In the young growing pig there undoubtedly would have been no such constancy of metabolism had protein not been a limiting factor in the diet. Indeed, Deighton's well nourished pig, 133 days old, weighing 29.5 kilos, showed a basal heat production of 1,483 calories per day, or twice that shown by our animal at the time of the same weight. It may be remarked that Coleman and Du Bois (7) attribute the high heat production of boys as due to the intensity of the growth processes, and they also point out that a higher basal metabolism exists during convalescence from typhoid fever when there is a renewal of lost cellular material. Perhaps the absence of a high metabolism in the pig is due to a restriction of the growth process.

III. THE INFLUENCE OF BENZOIC ACID.

The administration of 8 gm. of benzoic acid in the form of sodium benzoate had no influence upon the basal metabolism of the hog. The urinary analysis showed that only 0.281 gm. of glycine per hour was withdrawn from the metabolism of the pig by this procedure (Csonka, previous paper, p. 576). When the same dosage was administered during a period of high respiratory quotients the metabolism rose in an average of two experiments so that it measured 2 calories per hour above the level found on other days free from the influence of benzoic acid when the high quotients were found 18 hours after food ingestion. No significance is attached to this. The absorption of benzoic acid and the synthetic production of hippuric acid may therefore be accounted to be without influence upon the energy metabolism of the pig.

TABLE II.
The Influence of Benzoic Acid.

Food.	Experiment No.	Date.	R.Q.	CO ₂	O ₂	Urinary N.	Calories.		Increase over basal.	Weight.
							Indirect.	Direct.		
		1925-24		gm.	gm.	gm.			per cent	kg.
Benzoic acid, 8 gm.	34	Feb. 6	0.80	9.80	8.88	0.158	29.57	29.97	3.4	27.7
" " 8 "	6	Dec. 22	1.23	17.28	10.25	0.158	37.24	32.10	30.2	21.7
" " 8 "	17	Jan. 9	1.14	16.48	10.55	0.158	37.72	33.80	31.8	24.7
Average.....			1.19	16.87	10.40	0.158	37.48	32.95	31.0	
Hippuric acid, 10 gm.	32	Feb. 4	0.79	10.64	9.84	0.145	32.65	31.54	14.1	27.8
" " 10 "	37	" 11	0.76	9.21	8.85	0.145	29.10	31.41	1.7	27.6
Average.....			0.78	9.93	9.35	0.145	30.88	31.48	7.7	
Hippuric acid, 10 gm.	23	Jan. 18	1.19	16.02	9.76	0.396	34.55	36.67	20.8	26.0
" " 10 "	25	" 22	1.08	16.16	10.85	0.396	37.86	36.35	32.3	26.6
Average.....			1.14	16.09	10.31	0.396	36.21	36.51	26.6	

When 10 gm. of *hippuric acid* were given to the pig the heat production in one experiment rose only 2 per cent above the normal. When 10 gm. of hippuric acid were given during the high respiratory quotient periods there was an inappreciable increase in the heat production which averaged 0.8 calorie. The absorption and elimination of hippuric acid may therefore be accounted to be without influence on metabolism, thus resembling urea in this regard.

IV. GLYCINE AND BENZOIC ACID.

The administration of 10 gm. of glycine to the hog resulted in an average increase above the basal metabolism of 6 per cent, a very slight effect. When 10 gm. of glycine were given with 8 gm. of benzoic acid there was no increase whatever. Benzoic acid, uniting with the ingested glycine, prevented the specific dynamic action of the material.

Curiously enough, however, when the same materials were given at a time of high respiratory quotients 18 hours after the ingestion of starch, there was an average increase of 50 per cent above the level of the basal metabolism. The respiratory quotients averaged 1.17. The only other instance in which the metabolism reached this height was in the hours following the ingestion of 200 gm. of rice and 50 gm. of glucose when it rose 54 per cent, and the respiratory quotient was also 1.17.

TABLE III.
The Influence of Glycine and Benzoic Acid.

Food.	Experiment No.	Date. 1923-24	R.Q.	CO ₂	O ₂	Urinary N.	Calories.		Increase over basal.	Weight. kg.
							Indirect.	Direct.		
Glycine, 10 gm.....	8	Dec. 28	0.89	11.02	8.99	0.090	30.78	29.20	7.6	22.4
" 10 "	24	Jan. 21	0.92	11.00	8.67	0.090	29.89	32.36	4.5	26.0
Average.....			0.91	11.01	8.83	0.090	30.34	30.78	6.1	
Glycine, 10 gm., and benzoic acid, 8 gm....	39	Feb. 14	0.81	9.41	8.45	0.377	27.72	27.13	-3.1	27.7
The same diet.....	19	Jan. 12	1.17	19.62	12.17	0.279	43.49	40.62	52.0	25.2
" "	22	" 17	1.16	18.94	11.87	0.237	42.45	43.64	43.4	25.8
Average.....			1.17	19.28	12.02	0.258	42.97	42.13	50.2	

V. CASEIN, GLYCINE, AND BENZOIC ACID.

Casein, 43.7 gm., containing 6 gm. of nitrogen, was given to the pig and an increase of 12 per cent in metabolism was noted. The conditions were highly favorable for the retention of protein by the animal. When 43.7 gm. of casein, 10 gm. of glycine, and 8 gm. of benzoic acid were given, an average increase of 12 per cent was found from two widely disagreeing experiments. According to Csonka (previous paper, p. 572), when glycine unites with benzoic acid the protein of casein is as readily deposited in the body as when casein alone is given. On *a priori* grounds one would therefore expect to find the same metabolism in the two instances.

Moreover, four experiments done on successive days during a period of high respiratory quotients show that the metabolism after giving casein and benzoic acid, and the same plus glycine, is the same by the two procedures.

TABLE IV.
The Influence of Casein, Glycine, and Benzoic Acid.

Food.	Experiment No.	Date.	R.Q.	CO ₂	O ₂	Urinary N.	Calories.		Increase over basal.	Weight.
							Indirect.	Direct.		
		1923-24		gm.	gm.	gm.			per cent	kg.
Casein, 43.7 gm.	9	Dec. 29	0.95	12.18	9.29	0.120	32.21	30.38	12.6	22.4
" 43.7 " and benzoic acid, 8 gm	12	Jan. 3	1.07	15.17	10.35	0.212	36.43	32.66	27.3	23.4
" 43.7 " " " 8 " "	14	" 5	1.17	17.76	11.08	0.257	39.56	33.00	33.3	23.7
Average			1.12	16.47	10.72	0.235	38.00	32.83	32.8	
Casein, 43.7 gm., glycine, 10 gm., benzoic acid, 8 gm.	35	Feb. 7	0.77	11.13	10.48	0.344	34.29	35.28	19.9	27.4
Casein, 43.7 gm., glycine, 10 gm., benzoic acid, 8 gm.	46	" 26	0.79	9.97	9.15	0.344	30.01	29.61	4.9	27.8
Average			0.78	10.55	9.82	0.344	32.15	32.45	12.4	
Casein, 43.7 gm., glycine, 10 gm., benzoic acid, 8 gm.	11	Jan. 2	1.08	15.81	10.60	0.242	37.48	30.22	31.0	22.9
Casein, 43.7 gm., glycine, 10 gm., benzoic acid, 8 gm.	13	" 4	1.08	16.44	11.03	0.275	38.83	35.64	35.7	23.4
Average			1.08	16.13	10.82	0.259	38.16	32.93	33.4	

VI. GELATIN, GLYCINE, AND BENZOIC ACID.

Administration of 40.4 gm. of gelatin (6 gm. of N) to the pig increased the metabolism 8 per cent; giving the same plus 10 gm. of glycine, 10 per cent (as calculated from the basal metabolism of the following day); and after these two materials plus benzoic acid, 13 per cent. Benzoic acid exerted no pronounced influence.

In the experiments with high respiratory quotients the results obtained are extremely variable. One may, however, justly compare Experiments 20 and 21, done on successive days, in both of which gelatin and benzoic acid were given and in the former of which 10 gm. of glycine were also added. In the first of the two the respiratory quotient was 1.02 and the heat production 35.3 calories per hour; in the latter these figures were 1.05 and 35.4, respectively. Here again benzoic acid was without influence upon the heat production.

TABLE V.
The Influence of Gelatin, Glycine, and Benzoic Acid.

Food.	Experiment No.	Date.	R.Q.	CO ₂	O ₂	Urinary N.	Calories.		Increase over basal.	Weight.
							Indirect.	Direct.		
		1923-24		gm.	gm.	gm.			per cent	kg.
Gelatin, 40.4 gm.	3	Dec. 19	0.94	11.59	8.94	0.118	30.90	31.80	8.0	20.3
" 40.4 " and glycine, 10 gm.	40	Feb. 15	0.79	9.58	8.80	0.373	28.80	30.80	0.7*	27.8
" 40.4 " " benzoic acid, 8 gm.	4	Dec. 20	1.03	13.60	9.59	0.262	33.38	31.47	16.7	20.9
" 40.4 " " " 8 " "	5	" 21	1.02	12.09	8.63	0.262	29.98	30.85	4.8	21.2
" 40.4 " " " 8 " "	15	Jan. 7	1.11	17.18	11.22	0.204	39.86	36.11	39.3	24.0
" 40.4 " " " 8 " "	21	" 16	1.05	15.02	10.03	0.233	35.39	36.02	23.7	25.8
Average			1.05	14.47	9.87	0.240	34.65	33.61	21.1	
Gelatin, 40.4 gm., glycine, 10 gm., benzoic acid, 8 gm.	20	Jan. 15	1.02	14.11	10.10	0.204	35.28	32.47	23.3	25.2
Gelatin, 40.4 gm., glycine, 10 gm., benzoic acid, 8 gm.	36	Feb. 8	0.83	11.04	9.70	0.204	32.45	31.20	13.4	27.5

* When calculated from the basal metabolism of Feb. 16 this experiment shows an increase in metabolism of 10 per cent.

VII. THE PRODUCTION OF FAT FROM CARBOHYDRATE.

The question of the production of fat from carbohydrate has already been discussed by Lusk (8) in Paper XI of this series. It was set forth that in the production of 100 gm. of fat containing 950 calories from 270.06 gm. of glucose containing 997.2 calories there was a loss of heat equal to 5 per cent. Bleibtreu's formula, which was employed in these calculations, reads:

$$270.06 \text{ gm. glucose} = 100 \text{ gm. fat} + 115.45 \text{ gm. CO}_2 + 54.6 \text{ gm. H}_2\text{O}$$

Each liter of carbon dioxide liberated in this anaerobic reaction had a calculated heat value of 0.80 calorie. In the computation of the calories of metabolism by the indirect method, whenever the *non-protein respiratory quotient* exceeds unity, it is necessary to calculate the number of liters of carbon dioxide by which this quotient is exceeded, multiply by 0.8 calorie, and add the product to the calculated heat value of the protein and carbohydrate which are being oxidized.

From these data one may further compute that 1 liter of extra CO_2 elimination above the *non-protein respiratory quotient* of unity corresponds to the deposition of 1.7 gm. of fat which is derived from the metabolism of 4.80 gm. of glucose, or 5.06 gm. of starch. The values given in this paper are computed from these figures.

Table VII summarizes the results obtained in all the experiments in which high respiratory quotients were obtained, omitting those in which the addition of protein or glycine to the diet involved complications.

Of outstanding interest is the fact that high respiratory quotients were obtained 18 to 22 hours after the administration of a liter of milk and 300 gm. of corn-starch to the pig. This "standard diet" had the following composition.

	Protein.		Fat.		Carbohydrate.		Total.
	gm.	cal.	gm.	cal.	gm.	cal.	cal.
Milk, 1 liter.....	32	131	38	353	50	200	684
Corn-starch, 300 gm.....					270	1,107	1,107
Total.....							1,791

TABLE VI.
The Influence of Carbohydrate.

Food.	Experiment No.	Date.	R.Q.	CO ₂	O ₂	Urinary N.	Calories.		Increase over basal.	Weight.
							Indirect.	Direct.		
		1924		gm.	gm.	gm.			per cent	kg.
Corn-starch, 400 gm., glucose, 50 gm.....	28	Jan. 25	1.25	18.38	10.65	0.085	38.93	37.12	36.1	26.9
" 400 " 50 "	29	" 30	1.26	18.01	10.41	0.085	38.08	42.23	33.1	27.7
Average.....			1.26	18.20	10.53	0.085	38.51	39.68	34.6	
Rice, 200 gm.....	42	Feb. 19	1.11	16.07	10.52	0.082	37.63	36.92	31.5	28.9
" 200 " glucose, 50 gm.....	43	" 20	1.17	19.64	12.22	0.082	44.12	40.27	54.2	29.8
" 135 " benzoic acid, 8 gm.....	47	" 28	1.16	18.21	11.40	0.140	40.97	41.16	43.2	29.8

Food.		Date.	Experiment No.	Length of experiment.	Total calories.	R.Q.	Fat deposit per hr.		Calories of fat deposited (28.6 calories).
Extra on day before.	1 hr. before experiment.						gm.	cal.	
Hippuric acid, 10 gm.....	None.	1935-34 Jan. 23	26	3	36.5	1.18	2.36	22.4	
Starch, 400 gm., glucose, 50 gm.....	"	Feb. 1	31	1	35.5	1.22	2.68	25.5	
Rice, 200 gm., glucose, 50 gm.....	"	" 21	44	1	34.1	1.22	2.98	28.8	
" 135 "	"	" 29	48	2	35.3	1.17	2.10	20.0	
Average.....					35.0	1.20	2.53	24.0	83
Glycine, 10 gm., benzoic acid, 8 gm.....	Hippuric acid, 10 gm.	Jan. 18	23	3	34.6	1.18	2.43	23.1	
Glycine, 10 gm.....	" " 10 "	" 22	25	4	37.9	1.08	1.54	14.7	
Average.....					36.3	1.13	1.99	18.9	64
Benzoic acid, 8 gm.....	Benzoic acid, 8 gm.	Dec. 22	6	4	37.2	1.23	3.02	28.7	
Gelatin, 40 gm., benzoic acid, 8 gm.....	" " 8 "	Jan. 9	17	4	37.7	1.14	2.01	19.1	
Average.....					37.5	1.19	2.52	23.9	83
Rice, 200 gm.	Rice, 200 gm.	Feb. 19	42	3	37.6	1.11	1.55	14.7	51
Corn-starch, 400 gm. glucose, 50 gm.	" " 50 "	Jan. 25	28	2	38.9	1.25	3.40	32.3	
" 400 " " 50 "	" " 50 "	" 30	29	1	38.1	1.26	3.37	32.0	
Average.....					38.5	1.26	3.39	32.2	112
Rice, 135 gm., benzoic acid, 8 gm.	Rice, 135 gm., benzoic acid, 8 gm.	Feb. 28	47	4	41.2	1.17	2.47	23.5	82
" 200 " glucose, 50 gm.	" 200 " glucose, 50 gm.	" 20	43	3	44.1	1.17	2.61	24.8	87

The composition of the food was obtained from standard tables.

In Experiments 31, 44, and 48, in which the basal metabolism was obtained 18 hours after this diet, the pig had received carbohydrate food in quantity during the morning of the day before (see Experiments 43 and 47) from which some part of the energy for the day must have been derived.

It is notable that after giving rice and benzoic acid at 10.35 a.m. (Experiment 47) the respiratory quotient was 1.17, and that after following this with the standard diet at 5 p.m. the respiratory quotient the next day, between noon and 2 p.m., was again 1.17 (Experiment 48). In like manner, after giving 200 gm. of rice and 50 gm. of glucose at 10.30 a.m. the respiratory quotient was 1.17 between 11.30 a.m. and 2.30 p.m. (Experiment 43). The standard diet was taken at 5 p.m., and the next day between noon and 1 p.m. the respiratory quotient was found to be 1.22 (Experiment 44).

It is evident that after taking the "standard diet" alone high respiratory quotients may be noted 18 and more hours after the food intake, and Experiments 6 and 23, in which nothing else of food value was taken, bear added testimony to this.

According to the computations in the first four tabulated experiments in Table VII one may estimate that the pig retained 2.53 gm. of fat per hour or 60.7 gm. during 24 hours, an amount equal to 83 per cent of the calories of the basal metabolism. A calculation of the factors entering into the situation reveals the following relations.

Calories of the basal metabolism.....	686
" " " specific dynamic action of carbohydrate.....	154
" " materials deposited:	
Fat (60.7 gm.) from carbohydrate.....	576
" in milk (38 gm. \times 9.3).....	353
Protein (32 - 7* gm.) \times 4.1.....	102
Total.....	1,871
Calories in standard diet.....	1,791
* Protein metabolized.	

This computation shows an agreement within 5 per cent. More than half of the calories may be retained for growth. The calculation allows no margin of energy for the performance of

external work. That such work may be accomplished at the expense of the deposition of fat is, however, evident from the following experiment.

Experiment 44—Basal Metabolism.

Calories per hr.	R.Q.	Behavior.
34	1.22	Quiet.
38.2	1.13	Restless.
46.0	1.01	Much movement.

It is evident that the cost of muscular activity was at the expense of carbohydrate which would otherwise have been converted into fat.

The carbohydrate equivalent of the standard diet is 317.5 gm. of starch and, since 1 gm. of starch may produce a maximum of 0.336 gm. of fat, one may estimate a possible theoretical production of 107 gm. of fat from this source, whereas the figures given show an average production of 60.7 gm.

The maximum production of fat from carbohydrate occurred immediately after giving corn-starch and glucose together and reached a rate of 82 gm. per day (Experiment 28). In two experiments of this nature (Experiments 28 and 29) the deposition of fat in terms of energy equivalents was 112 per cent above the basal metabolism. It represented a production of fat from carbohydrate which was 77 per cent of the possible maximum.

It is evident that the pig is quite a remarkable fat-producing machine, the process being vigorously continued for 21 or more hours after the ingestion of a single meal rich in starchy food.

Comparing the results presented here with those obtained by Meissl and Strohmer (9) many years ago, one may calculate the following.

	Weight.	Fat from carbohydrate.	
		Per day.	Per kg.
	kg.	gm.	gm.
Our pig.....	28	61	2.1
Pig of Meissl and Strohmer.....	140	310	2.2

The results after giving benzoic acid to the pig show no influence upon either the level of the total metabolism or the function of fat production from carbohydrate.

As previously shown by Lusk (8), the results demonstrate that the height of the total metabolism is not proportional to the height of the respiratory quotient. The process of the formation of fat from carbohydrate requires little energy.

The *alcohol checks* covering the period in which these experiments were accomplished have already been published in Table XII of Paper XXVI of this series (10).

VIII. SUMMARY.

1. A pig 11 weeks old and 20 kilos in weight at the beginning, and nearly 27 weeks old and 31 kilos in weight at the end of the period of experimentation, manifested an average basal metabolism of 28.6 calories throughout the period with a plus or minus variation of 3 per cent (one low observation excepted).

The protein element in the pig's diet was notably restricted, thereby giving little play of the growth impulse. The stunted pig appeared perfectly healthy throughout the experimental period.

2. The heat loss by vaporization of water was 20 per cent of the whole.

3. The synthesis of benzoic acid with glycine to form hippuric acid has no certain effect upon the heat production under very varied conditions of diet.

4. The ingestion of 10 gm. of hippuric acid has no influence upon the heat production.

5. After administering 300 gm. of corn-starch and 1 liter of milk at 5 p.m. the process of formation of fat from carbohydrate was vigorously continued for 21 hours or more.

6. More than half of the calories of the diet could be retained for growth.

7. The maximum amount of fat manufactured from carbohydrate and retained by the pig during an hour amounted to 3.4 gm. or at the rate of 82 gm. per day and 77 per cent of the possible maximum. The respiratory quotient reached 1.25. The average retention of such fat was 2.53 gm. per hour or 61 gm.

per day, which was over half (56 per cent) of the amount theoretically derivable from the carbohydrate administered.

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ON THE NATURE OF BLOOD SUGAR.

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In a paper "On the nature of the sugar in blood," Winter and Smith (1) have recently advanced the interesting suggestion that the sugar in normal blood is the highly reactive isomer γ -glucose while in cases of severe diabetes the blood sugar is in the relatively stable α - β form.

The experimental evidence on which this hypothesis is based consists in the observation that deproteinized extracts of normal blood when examined polariscopically over a period of 3 days show a progressive downward rotation, and at the same time the power of these extracts to decolorize potassium permanganate decreases. It was also noted that sugar in the extracts as determined by polarization was invariably lower than when the analysis was made by titration (Bertrand method). On the other hand deproteinized extracts of blood taken from severe diabetics were found to show the phenomena just enumerated, either not at all, or in a greatly lessened degree. In a later communication (2) Forrest, Smith, and Winter have published further results confirming their earlier work on diabetics, and have also given an account of work on these patients treated with insulin, which leads them to believe that after the therapeutic use of this substance the blood sugar of the diabetic contains a greater proportion of the normal form of reducing sugar than before treatment, while in a third communication (3) Winter and Smith present a brief note on work done on rabbits made hyperglycemic by means of epinephrine injections, in which the statement is made that in this condition the mixture of sugars present in the blood would appear to be similar to that existing in diabetic blood before the injection of insulin. As a result of the

above work Winter and Smith have suggested the hypothesis that the cause of diabetes is the absence or inactivation of an enzyme whose function is to transform the relatively stable α - β -glucose into the exceedingly unstable γ form. The above mentioned hypothesis has been condemned on theoretical grounds by Hewitt (4) and by Macleod (5), neither of whom has undertaken a repetition of the work of the first named investigators. Such a repetition has, however, been made in Macleod's laboratory by Eadie (6), who has worked with the blood of normal rabbits and dogs, and with the blood of rabbits to whom epinephrine or epinephrine and insulin had been administered. The experimental results presented by this investigator can in our opinion be scarcely considered as confirming the observations of Winter and Smith, although Eadie himself refrains from any attempt at an interpretation of his work.

Work on the problem has recently been published by van Creveld (7), who, instead of employing chemical methods for the removal of protein, used for his experiments the aqueous humor of the eye and ultrafiltrates of serum and of artificial transudates. His experiments with this material led him to conclude that it was impossible to determine with certainty the existence of a possible mutarotation with the normal unconcentrated eye-chamber liquid, and after subconjunctival injection of adrenalin the aqueous humor "does not show mutarotation of any importance and that the optical value under these conditions closely corresponds to the reduction value. Reduction and optical value of serum-ultrafiltrate, of concentrated serum-ultrafiltrate, of aqueous humour and of concentrated aqueous humour, compared during four to five successive days, may remain the same from the beginning of the experiment."

A repetition of the work of Winter and Smith has also been made by Visscher (8), who states that he was able to corroborate their observations with normal blood, but found that the same difference that these investigators found to exist between normal and diabetic blood could be obtained by varying the H ion concentration of the deproteinized extract from normal blood. If the filtrate was nearly neutral it behaved like normal blood, but if strongly acid its behavior resembled that of diabetic blood.

Our work on the problem was undertaken without knowledge of the publication of Eadie and was largely completed before the appearance of the communications of van Creveld and of Visscher. As our experiment, while giving the same general results as have been described by these investigators, has led us to somewhat different conclusions it has seemed best to publish them at this time.

Procedure.

Experiments have been carried out on known amounts of glucose diluted to the concentration existing in normal and in diabetic blood and mixed with such amounts of inorganic salts and of the various non-protein nitrogenous constituents of blood as exist normally in this fluid. Two such solutions were used for this work which differed only in the fact that one contained creatine and creatinine and in the other these substances were omitted.

The composition of these stock solutions were as follows:

	Solution 1.	Solution 2.
	mg. per 100 cc.	mg. per 100 cc.
Urea.....	30	30
Alanine.....	60	60
Creatinine.....	0	2
Creatine.....	0	6
NaCl.....	600	600
KCl.....	400	400
MgSO ₄	15	15
KH ₂ PO ₄	24	24

Our work on animals has been carried out on the blood of rabbits who were stunned by a blow on the head and then quickly decapitated, on blood obtained from the carotid artery of fasting dogs, and on beef blood secured from the slaughter-house.

Our blood samples were precipitated by the Folin-Wu method, the filtrate was evaporated under reduced pressure, and the residue subsequently extracted twice with 85 per cent alcohol, again evaporated to dryness under reduced pressure, and subsequently redissolved in water: all exactly as described by Winter and Smith.

As prescribed by these authors the processes of evaporation, filtrations, etc., were carried through with the greatest possible speed, and in no case was this part of the experiment extended over a period of more than 6 hours, while many of the experiments were complete in 3 or 4 hours. In order to obtain the concentrated extracts in a condition sufficiently clear for polariscopic work it was necessary to pass through a filter prepared ~~section~~ of filter paper supported on an asbestos mat, the ~~v. (5)~~ (5), contained in a Gooch crucible. In a few cases (noted in the protocols given below) the addition of a small amount of alumina cream was found essential for efficient clarification. The determination of reducing sugar in the original blood was made by the method of Folin and Wu (9) and in the concentrated protein-free extracts by the procedure of Shaffer and Hartmann (10).

Determinations of H ion concentrations were made with the colorimetric method by the use of buffer solutions which had been standardized electrometrically. Tests of the ability of the blood extracts to decolorize potassium permanganate were made by allowing 0.01 N potassium permanganate to fall drop by drop into 1 cc. of the liquid which had previously been heated to about 50°C.

During the period of storage the polariscope tubes were kept in the dark room in which the readings were made, which was found to have a temperature which varied only between 19° and 21°C.

In our investigations we used a Schmidt and Haensch 3-field instrument of the Lippich type, reading direct to 0.01° and Schmidt and Haensch 2 and 1 dm. tubes. Readings were taken at 19-21°C., and to preserve the dark adaptation of the observer's eye as far as possible, scale readings on the instrument were made with a small shielded flashlight. Preliminary experiments indicated that sodium light was not sufficiently intense to allow adjustment of the field of the instrument within the observer's known error on stable aqueous glucose solutions, and since the determination of whether or not any mutarotation occurs in the glucose solution was of more importance in the problem under investigation than the accurate determination of the ratio between the polarimetric and copper-reducing values we have abandoned the use of sodium light entirely and in all our experiments have

used a 100 c.p. electric bulb with a compact spiral filament as a source of light, and as a filter a 3 cm. layer of 3 per cent $K_2Cr_2O_7$. In Table I we have collected the results obtained with blood. An inspection of these figures shows:

1. That the percentage of glucose in the blood extracts is invariably higher when determined by copper reduction than ne. calculated from the optical rotation, a finding which has

noted by earlier investigators. Furthermore, it would appear to us that such difference in results obtained by the two methods is only what may be expected in view of the fact that there are undoubtedly present in blood substances other than glucose which are responsible for a part of the optical rotation and of the reducing power of deproteinized extracts.

2. We are unable to confirm the findings of Winter and Smith regarding a loss or lessened activity for decolorization of potassium permanganate by the blood extracts after standing for several days, as in only one case (Experiment 14) was any loss of reducing power observed.

3. Our results as far as they go do not give confirmation to the suggestion of Visscher regarding the effect of hydrogen ion concentration on the changes in optical rotation and on reduction. It may be said, however, that so far Visscher has only published his work in abstract form in which no specific data regarding the degree of acidity is given, so that it is quite possible, that the variations in hydrogen ion concentration used by us, are not of the same magnitude as those employed by this investigator.

4. In looking over our figures it will be seen that in certain cases, as for example Experiments 1, 2, 20, etc., there was no decrease in rotation while in other experiments, *viz.* Nos. 15 and 11, a distinct reduction was observed. In considering the cause of this dissimilarity of conduct in blood extracts, all of which had been prepared by the same technique, it was noted on critical examination of the protocols of each experiment that in every case in which a drop in rotation was observed this fall coincided with the appearance of molds in the liquid contained in the polariscope tube. This finding led us to substitute sodium or potassium fluoride alone or mixed with thymol, for the potassium oxalate heretofore used as an anticoagulant, with the result that in our subsequent experiments no drop in rotation was observed. Ex-

TABLE I—*Ex.*

Anticoagulant.	Blood used.	Blood.	Daily readings in degrees.					
			1	2	3	4	5	6
		<i>mg. per 100 cc.</i>						
assium oxalate.	Dog.		0.16	0.17	0.16	0.17	0.16	0.16
" "	"	158	0.20	0.22	0.20	0.20		
" " and thymol.	"	200	0.20	0.22	0.24	0.22	0.21	0.11
" oxalate.	"	200	0.15	0.19	0.18	0.11	0.10	0.11
" "	Rabbit.	—	0.19	0.17	0.15	0.16	0.15	
" "	Beef.	107	0.03	0.03	0.05	0.00	-0.03	
" "	"	107	0.03	0.03	0.00	0.03	0.01	
ium fluoride.	"	107	-0.05	0.00	-0.02	-0.05		
" "	"	107	0.00	-0.03	-0.04			
" "	Dog.	158	0.34	0.35	0.35	0.36	0.33	
assium fluoride.	"	132	0.00	0.00	0.10	0.14	0.15	0.11
" "	"	132	0.30	0.32	0.31	0.28	0.30	0.3
ium fluoride.	"	200	0.19	0.17	0.17	0.19	0.17	
" " and thymol.	"	200	0.29	0.27	0.30	0.28	0.30	
assium oxalate.	Rabbit.	362	0.75	0.75	0.79	0.73	0.71	0.6
" "	"	416	0.40	0.38	0.41	0.36	0.40	0.4

otherwise specified all readings made in 2 dm. tubes.

periment 12 is unique in that the polariscope readings increased as the solutions become older, a result which we are unable to explain.

Results on the same order as those with blood extracts were obtained when we worked with solutions of glucose, salts, and nitrogenous extractives. The figures on this material are collected in Table II, and it will be seen that here, as in the case of the blood extracts, the percentage of glucose as calculated from the polariscope readings is invariably less than when determined by titration.

KMnO ₄ decolor- ized drops 0.01 N.		pH		Remarks.*
Initial.	Final.	Initial.	Final.	
				No mold.
		4.6	4.4	" "
13	8	5.2	5.0	" "
12	12	3.2	3.5	Small mold formation on 4th day.
12	12	4.6	4.9	" " " " 3rd " Readings made in a 1 dm. tube
16	16	5.4	5.4	Al(OH) ₃ to clarify. No mold.
16	16	4.0	4.0	" " " " "
15	15	6.4	6.4	" " " " "
13	13	1.8	1.8	" " " " "
4	4	6.2	6.4	No mold formation.
5	4	4.5	4.6	" " " This experiment unique in that reading increased.
13	13	5.1	5.1	No mold formation.
4	4	6.6	6.5	Al(OH) ₃ added to clarify, considerable protein present. N mold formation.
4	4	6.6	6.6	Al(OH) ₃ added to clarify. No mold formation.
8	8	4.6	4.6	Received 1 cc. of a 1:1,000 solution of epinephrine subcutaneously at 8.00 a.m. and a second injection of 2 cc. at 8.30. Blood collected 30 minutes later. No mold formation. Weight of animal 3.3 kg.
15	15	4.6	4.2	Received the same dose of epinephrine as in Experiment 15. Weight of animal 3.2 kg. No mold.

In the case both of blood extracts and of solutions we have observed that, while a fall of rotation is noted to be coincident with the appearance of a growth of mold, the copper reduction value does not always change (as for example in the case of Experiments 15, 4, and 9, although sometimes it does as in Experiments 3 and 7. In our opinion the first mentioned result can only be explained on the assumption that the mold is here nourished by some substance other than glucose.

TABLE II.
Experiments with Solutions.

Experiment No.	Initial glucose added.	Daily readings in degrees.						Glucose.				pH of extracts.		Remarks.	
								Initial by:		Final by:					
								1	2	3	4	5	6		Polarization.
	mg.														
2	100	0.17	0.15	0.15	0.15			0.16	0.24	0.14	0.24	6.9	5.9	Salt solution 1.	
3	100	0.26	0.25	0.25	0.23	0.19		0.25	0.26	0.18	0.22	5.3	4.4	" " 1. Mold developed on 4th day.	
4	200	0.53	0.51	0.52	0.44			0.50	0.53	0.42	0.52	6.0	4.3	Salt solution 1. Mold developed on 4th day.	
7	400	1.45	1.46	1.47	1.48	1.41	1.27	1.38	1.40	1.21	1.33			Salt solution 2 +1.5 gm. potassium oxalate. Mold developed on 5th day.	
8	400	1.29	1.27	1.27	1.25	1.26	1.26	1.23	1.38	1.20	1.30			Salt solution 2 +1 gm. sodium fluoride. Very small mold formation on 6th day.	
9	400	1.15	1.14	1.13	0.99	0.95	0.97	1.10	1.30	0.92	1.25			Duplicate of Experiment 7. Mold developed on 4th day.	
10	400	1.26	1.28	1.26	1.24	1.25	1.21	1.20	1.45	1.15	1.43			Duplicate of Experiment 8. Mold developed on 6th day	

SUMMARY.

In a repetition of the work of Winter and Smith on the nature of blood sugar, we have attacked the subject from the three points which these authors consider as giving evidence in favor of the theory regarding the presence of γ -glucose in normal blood: first a decreased power on the part of the blood extracts to decolorize potassium permanganate solutions when these extracts are kept for 3 days at laboratory temperature; second a progressively decreasing power to rotate polarized light; and third a difference between the percentage of glucose in these extracts when calculated from the polariscope reading and from titration.

As regards changes in ability to decolorize potassium permanganate we have been entirely unable to confirm the findings of Winter and Smith as no decrease in reducing value occurred in any of our extracts.

As regards polariscopic observations, we have found that the gradual fall in rotation in extracts from oxalated blood, and observed for a period of 3 or more days, was coincident with the appearance of mold in the solutions, when sodium or potassium fluoride either with or without the addition of thymol was substituted as an anticoagulant for potassium oxalate, the changes in rotation described by these investigators were not observed either in blood extracts or in "artificial blood extracts" prepared from glucose, salts, and nitrogenous extractives.

When a comparison was made of the percentage of glucose in our blood extracts calculated from the polariscopic readings and from titration (Shaffer-Hartmann method) it was found, as was pointed out by Winter and Smith, that higher results were obtained by the latter method. It was also noted, however, that a similar result was obtained with our "synthetic" blood extracts prepared from glucose, salts, and nitrogenous extractives and subjected to a distillation and extraction process exactly similar to the one used on blood.

Our results, therefore, make it seem unjustifiable to consider that the experiments of Winter and Smith furnish proof of the existence of γ -glucose in normal blood.

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THE BIOLOGICAL VALUE FOR MAINTENANCE AND GROWTH OF THE PROTEINS OF WHOLE WHEAT, EGGS, AND PORK.

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The experiments to be reported in this paper were planned with two objects in view: one, to obtain quantitative information concerning the relative biological values for maintenance and growth of the proteins of whole wheat, egg, and pork, as determined by investigation of the utilization by growing rats of their content of total nitrogen, and the other, to test out the method adopted in this laboratory (1) of conducting such investigations and of interpreting the results secured. It was desired, in particular, to discover whether the order in which a series of foods was tested influences in any way the biological values obtained, and whether the utilization of food proteins is increased if fed immediately after a 10 day period in which the experimental subjects are greatly depleted in nitrogen by subsistence on a low nitrogen diet.

A litter of nine rats, weighing from 70 to 80 gm. each, was divided into two groups, one of five and one of four rats. The first group was fed the different rations in the following order: protein-free, wheat, egg, pork, and protein-free. The order of feeding for the second group was as follows: protein-free, egg, pork, wheat, and protein-free. The feeding periods were of 10 days duration, on the last 7 days of which collections of feces and urine were made. The only change in procedure from that already reported was that the rat, instead of resting directly on the filter paper mat in the bottom of the crystallizing dish, was supported about $\frac{1}{2}$ inch above the filter paper on a circular disc of $\frac{1}{4}$ inch mesh wire screen, resting on a bent heavy glass rod (see Fig. 1). By this means consumption of feces and filter paper by the rat was practically entirely avoided.

To maintain the appetite of the rats throughout the experiment, each rat was given, aside from its food, 25 mg. daily, in all periods, of a commercial vitamin B product (yeast vitamin-Harris powder), prepared according to the method of Osborne and Wakeman. This amount contained by analysis 2.34 mg. of nitrogen, 16 per cent of which was free amino nitrogen, and 28 per cent free and combined amino nitrogen. The possible vitiating effect of this small amount of nitrogen on the estimations of biological value has been discussed elsewhere (2). The figures given in the tables for nitrogen intakes and biological values do not include or involve in any way this amount of nitrogen.



FIG. 1. Equipment used in metabolism investigations on rats.

The protein content of the rations to be tested in this experiment was approximately 8 per cent; *i.e.*, sufficiently low so that, even on the best protein, maximum retention of nitrogen would hardly be attained. Their composition is given in Table I.

The egg, pork, and wheat were dried at a low temperature; the egg and pork were subsequently partially extracted with ether to remove the excess fat. The Osborne and Mendel salt mixture (3) was used. With the protein-free ration, all the ingredients, except the fats, were mixed with water and steam-cooked until the starch was thoroughly dextrinized, the fats were then mixed in, the mixture was dried at a low temperature, and ground in the laboratory mill. The other rations were not cooked. The dried rations were weighed out for the rats each day, and mixed with water to

prevent scattering as far as possible. Residues remaining in the food cups were removed each day, dried, and the total weekly residue was subtracted from the total food offered to obtain the average daily intake. Except in the last period of feeding on the protein-free diet, no appreciable scattering occurred. In the last period, in which the scattered food was contaminated with urine, the nitrogen content of the food residues was determined in order to get the total excretion of urinary nitrogen.

The results and calculations pertaining to the first group of five rats are given in Table II and those for the second group of four rats are given in Table III.

The estimated "metabolic nitrogen" in the feces for all rats on the egg and pork rations was larger than the total fecal nitrogen

TABLE I.
Percentage Composition of Rations.

	Protein-free ration.	Egg ration.	Pork ration.	Wheat ration.
Dried wheat.....				67.7
" egg.....		11.5		
" pork.....			10.2	
Starch.....	76	64.5	65.8	15.3
Sucrose.....	10	10	10	5
Butter fat.....	8	8	8	8
Cod liver oil.....	2	2	2	2
Salt mixture.....	4	4	4	2
Nitrogen.....	0.055	1.369	1.368	1.303

actually obtained, so that there is no reason for doubting that complete absorption of dietary nitrogen was attained in these cases. As in most of our experiments, the endogenous losses in the urine per 100 gm. of body weight decreased from the first to the last period. In estimating the food nitrogen in the urine in the intervening periods, therefore, it was assumed that this decrease was linear. The biological values, included in the last column of the table, represent the amount of dietary nitrogen consumed minus the estimated losses in feces and urine, in percentage of the absorbed nitrogen.

For both groups of rats it is evident that a clear distinction existed between the protein values of the three foods investigated. The outstanding superiority of the nitrogen of egg is notable.

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TABLE II.

Metabolism Data and Computations for Group of Five Rats.

Rat No.	Initial weight.	Final weight.	Daily food intake.	Daily nitrogen intake.*	Daily fecal nitrogen.	Daily urinary nitrogen.	Metabolic nitrogen in feces per gm. food.	Endogenous nitrogen in urine per 100 gm. body weight.	Food nitrogen in feces.	Absorbed nitrogen.	Food nitrogen in urine.	Total food nitrogen retained.	Biological value.
Period 1. Protein-free ration.													
	gm.	gm.	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	per cent
201	68	64	4.77	2.6	6.5	18.3	1.37	27.8					
202	75	69	5.20	2.7	8.0	20.6	1.54	28.6					
203	80	72	4.82	2.7	8.2	21.4	1.71	28.2					
204	80	73	4.87	2.7	7.7	23.8	1.59	30.3					
205	70	65	4.85	2.7	8.7	20.2	1.79	29.7					
Period 2. Wheat ration.													
201	73	80	7.53	97.9	18.4	53.9			7.6	90.3	34.3	56.0	62
202	76	86	7.68	99.8	19.7†	53.5			7.3	92.5	31.3	61.2	66
203	80	88	8.27	107.5	22.4	60.2			8.3	99.2	38.0	61.2	62
204	77	87	7.64	99.3	21.3	54.4			8.7	90.6	32.3	58.3	64
205	69	79	8.48	110.2	22.2	59.2			6.9	103.3	39.7	63.6	62
Period 3. Egg ration.													
201	90	109	7.93	108.6	7.8	30.3			0	108.9	7.2	101.4	93
202	95	111	7.95	108.9	9.6	29.2			0	108.9	.3	106.6	98
203	94	112	7.91	108.4	9.9	31.4			0	108.4	.2	102.2	94
204	92	109	7.89	108.1	9.5	31.4			0	108.1	.4	100.7	93
205	90	107	8.00	109.6	9.5	31.8			0	109.6	9.0	100.6	92
Period 4. Pork ration.													
201	114	123	7.98	109.2	11.2	60.0			0	109.2	35.4	73.8	68
202	118	124	7.98	109.2	11.5	54.9			0	109.2	24.9	84.3	77
203	113	124	7.78	106.4	9.5	55.7			0	106.4	28.8	77.6	73
204	111	120	7.95	108.8	9.9	56.3			0	108.8	32.5	76.3	70
205	113	121	7.99	109.3	11.9	64.6			0	109.3	41.7	67.6	62

* Exclusive of the nitrogen contained in the yeast extract given as a source of vitamin B. This amounted to 2.3 mg. per rat per day.

† This sample of feces was lost during analysis. The figure given is based upon the average digestibility of wheat nitrogen for the other four rats.

TABLE II—*Continued.*

Rat No.	Initial weight.	Final weight.	Daily food intake.	Daily nitrogen intake.*	Daily fecal nitrogen.	Daily urinary nitrogen.	Metabolic nitrogen in feces per gm. food.	Endogenous nitrogen in urine per 100 gm. body weight.	Food nitrogen in feces.	Absorbed nitrogen.	Food nitrogen in urine.	Total food nitrogen retained.	Biological value.
Period 5. Protein-free ration.													
201	117	107	5.30	2.9	8.8	20.6	1.66	18.4					
202	116	106	5.14	2.8	9.6	26.0	1.87	23.5					
203	114	104	5.95	3.3	10.1	22.5	1.71	20.7					
204	118	110	5.74	3.2	10.6	19.8	1.85	17.3					
205	116	108	5.80	3.2	11.0	18.2	1.89	16.2					

Both groups of rats gave closely agreeing biological values, averaging 93. Considering the level of protein intake, this is the highest value that we have thus far obtained for any food, surpassing even that of milk. The superiority of pork protein (nitrogen) over that of wheat protein, while not great, seems established. For eight of the nine rats, the pork value was higher than the wheat value, while for one rat identical values were obtained. The average values for all rats were 74 for pork, and 65 for wheat. In an earlier experiment, the detailed results of which need not be considered, average values of 74 for pork and 70 for wheat were obtained with a group of five rats, the rations being very similar to those described in Table I.

In considering the protein value of any variety of meat, it should be remembered that a considerable fraction of the nitrogen of meat, about 13 per cent (4), is in the form of water-soluble extractions, much of which is probably of little biological value; also, that hemoglobin and the proteins of connective tissue, present in variable amounts in all meat, are of inferior biological value. In all probability the proteins of the muscle tissue itself are of superior value in nutrition, and, considering the high content of meat in easily digestible protein, the only moderately high biologi-

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TABLE III.

Metabolism Data and Computations for Group of Four Rats.

Rat No.	Initial weight.	Final weight.	Daily food intake.	Daily nitrogen intake.*	Daily fecal nitrogen.	Daily urinary nitrogen.	Metabolic nitrogen in feces per gm. food.	Endogenous nitrogen in urine per 100 gm. body weight.	Food nitrogen in feces.	Absorbed nitrogen.	Food nitrogen in urine.	Total food nitrogen retained.	Biological value.
Period 1. Protein-free ration.													
	gm.	gm.	gm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	per cent
206	81	71	4.18	2.3	6.7	22.5	1.60	29.7					
207	80	71	4.78	2.6	10.4	23.8	2.17	30.2					
208	72	67	4.14	2.3	5.7	20.8	1.38	29.9					
209	78	72	5.16	2.9	7.9	21.7	1.52	28.9					
Period 2. Egg ration.													
206	80	102	7.75	106.1	7.4	32.5			0	106.1	8.1	98.0	92
207	81	101	7.99	109.4	8.9	32.3			0	109.4	8.2	101.2	92
208	75	93	7.57	103.6	9.4	31.9			0	103.6	9.9	93.7	90
209	84	106	7.97	109.1	8.3	32.8			0	109.1	7.4	101.7	93
Period 3. Pork ration.													
206	108	117	8.00	109.4	8.3	52.2			0	109.4	26.9	82.5	75
207	108	117	8.00	109.4	6.8	52.7			0	109.4	25.6	83.8	77
208	100	111	8.00	109.4	9.3	53.1			0	109.4	29.5	79.9	73
209	110	127	8.00	109.4	5.2	42.0			0	109.4	13.0	96.4	88
Period 4. Wheat ration.													
206	122	134	8.98	117.2	19.8	62.8			5.2	112.0	35.9	76.1	68
207	125	136	9.00	117.3	18.0	62.2			3.2	114.1	37.4	76.7	67
208	117	129	8.99	117.3	22.1	62.2			6.7	110.6	39.3	71.3	64
209	130	141	8.97	117.2	18.5	61.8			4.0	113.2	31.7	81.5	72
Period 5. Protein-free ration.													
206	129	118	5.00	2.7	8.1	22.4	1.63	18.2					
207	130	123	5.98	3.3	8.7	19.2	1.46	15.2					
208	124	116	6.01	3.3	11.0	17.7	1.83	14.9					
209	134	125	5.85	3.2	9.7	25.8	1.65	20.0					

* Exclusive of the nitrogen contained in the yeast extract given as a source of vitamin B. This amounted to 2.3 mg. per rat per day.

cal values here obtained for its *total* nitrogen do not constitute an argument against placing meat, along with eggs and milk, as an excellent source of protein in practical dietetics. The following computations of the "net protein" values (5) of these foods illustrate this fact very well.

While the relation between the biological values for the protein of the three foods investigated was the same for both groups of rats, the values for pork and for wheat obtained for the group of four rats averaged from 5 to 7 points higher than those for the group of five rats. However, this discrepancy does not seem to be related to the order in which the foods were fed. In particular, the values obtained in periods immediately following the feeding of low nitrogen diets, were not higher than corresponding values obtained in periods not so situated.

TABLE IV.

The Net Protein Values of Pork, Eggs, and Wheat at an 8 Per Cent Level of Intake.

Food.	Total protein (N \times 6.25).	Percentage losses.		Net protein content.
		In digestion.	In metabolism.	
Eggs.....	13.4	0	7	12.5
Ham, fresh lean.....	19.8	0	26	14.6
Wheat.....	12.5	9	30	7.6

Individual differences in the utilization of dietary nitrogen in metabolism are clearly evident. Thus, Rats 202 and 209 gave consistently high values for all foods, while Rats 205 and 208 gave low values consistently.

CONCLUSIONS.

1. The average biological values found for the proteins (N \times 6.25) of eggs, pork, and wheat at an 8 per cent level of intake were, respectively, 93, 74, and 67, the latter two figures including earlier comparable determinations not reported in detail in this paper. The differences indicated by these averages are of high significance, since they were obtained for each of the nine rats used in the experiment, with one exception noted in the text.

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2. Though the biological value of its total nitrogen is thus not much greater than that of the nitrogen of whole wheat, lean pork must still be ranked among the best protein foods, because of its high "net protein" value. Based on average determinations of the crude protein content of these foods, and the percentage losses in digestion and metabolism indicated by the results of the experiments here reported, the content of net protein of egg is 12.5 per cent, of lean pork, 14.6 per cent, and of whole wheat, 7.6 per cent.

3. The order in which different foods are tested for their protein value in nutrition has no appreciable effect on the results obtained. In particular the testing of a food after a period of nitrogen depletion of the experimental animal, does not tend to increase the biological value of its protein.

4. Considerable and consistent individual differences may be demonstrated among individual rats in the utilization of dietary nitrogen in metabolism.

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CHANGES IN THE SERUM PROTEIN STRUCTURE OF RACHITIC RATS WHILE FED WITH COD LIVER OIL.

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A considerable degree of knowledge is accumulated about the albumin-globulin ratio in the serum. It is now known that this ratio is influenced by several factors and changes under certain pathological conditions. It is, moreover, recognized that a definite relationship exists between tissue destruction and amount of globulin in the serum, and that the latter is increased when an abnormal breaking down of tissue occurs. Such conditions as malignant tumors (1), chronic inflammatory diseases (2), starvation (3), radiation with chemically active light (1), chronic infections (4, 5), *viz.* lues and progressive tuberculosis, cause a definite increase in the serum globulin. Only a few conditions are, however, known which result in a decrease of the serum globulin—as pernicious anemia (6) and ascent into high altitudes (6).

According to R. Mond (7), ultra-violet light causes in protein solutions *in vitro* an increased viscosity and a shifting of the stability; namely, the coagulation temperature and coagulation zone of the globulins are increased whereas the albumin exerts a change in the opposite direction. Several authors have observed the transformation of albumin into globulin under the influence of such simple stimuli as heat (8), dialysis (9), and long standing (10).

The investigations reported in this paper were carried out to ascertain what, if any, alterations occurred in the structure and relationship of the proteins of the serum of rachitic animals during the healing of rickets under the influence of cod liver oil. These determinations have been made with young rats 60 to 80 days old, weighing 80 to 150 gm. The animals were made rachitic by

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restricting them to the "line test" diet, No. 3143. This diet has the following composition.

<i>Ration 3143.</i>		<i>gm.</i>
Maize.....		33.0
Wheat		33.0
" gluten.....		15.0
Gelatin.....		15.0
NaCl.....		1.0
CaCO ₃		3.0

The effect of cod liver oil and the extent of healing were studied histologically. The relation of albumin to globulin in the serum was determined from data obtained by the determination of serum viscosity and refraction by interpolation with the aid of Nægeli's chart. This chart is based upon the definite relation, established by Hayder and Rohrer, among the three factors: viscosity, protein percentage, and albumin-globulin ratio. They established the viscosimetric curves of albumin and globulin solutions of different concentrations. Viscosity was measured with the viscosimeter of Hess and the concentration with Pulfrich's refractometer. The albumin and globulin curves determine a field in which the refractivity is plotted against the viscosity and which includes all possible rates of any mixture of albumin and globulin. By means of this chart it is possible to interpret an unknown mixture of albumin and globulin when the viscosity and the refraction of the serum have been determined. A discussion of this chart is found in Nægeli's book (11) and in a paper of Bircher on viscosity (4).

Viscosity is expressed in relation to the viscosity of distilled water at 20°C. In this series a viscosimeter of Hess was used.

Refractivity is expressed in the units of Pulfrich's immersion refractometer.

The accuracy of the method used is fully discussed in the textbook of Nægeli and the literature which has accumulated on the method (12-15).

There are two theories as to the interpretation of the results of determinations of the viscosity and refractivity of the blood serum. Rohrer, Nægeli, and Bircher believe that the viscosity of serum is dependent on the relation of albumin and globulin and on the protein concentration. Neuschloss and Ellinger explain the changes

in the "specific viscosity" or "viscosity factor" by changes in the hydration of the proteins. This they believe results from the presence in them of some pathological substances in the serum which alters both the viscosity and the speed of ultrafiltration.

It is not the purpose of this investigation to discuss this problem, which is to be solved only on a basis of special colloid chemical data which are not available now. The changes which were found in the properties of the serum examined we expressed in this paper in terms of the percentage content of albumin. An increase in albumin is a sign of an increase in the stability of the serum proteins and their compounds, since the globulins have a more hydrophil character than albumins and are therefore more unstable,

TABLE I.

Average Figures of Viscosity, Refractivity, and Albumin Percentage of Serum.

Specimen.	Viscosity.	Refractivity.	Albumin.
		<i>Pulfrich units</i>	<i>per cent</i>
Normal adult rat.....	1.761	64.50	80.70
Young rats of 70 to 90 gm.....	1.505	54.70	81.07
Rachitic rats (80 to 150 gm.) showing advanced healing (+++).....	1.731	60.19	72.09
Rachitic rats of the same weight with beginning healing (+ or ++).	1.640	59.96	83.52
Rachitic rats of the same weight.....	1.625	59.80	86.40

since stability is an expression of the affinities of the colloid particles for the solvent.

Globulin sensitizes the positively charged Congo red particle and the negative night blue equally to precipitation by alkalis, and since calcification of the bone is a process of precipitation of lime salts in them, this possibility of the sensitization of the blood calcium to precipitation must be taken into consideration in discussing the mechanism of this process.

Tables I to V show the results of the determinations which were made in carrying out these studies. From the data presented the following conclusions can be drawn.

1. The viscosity of the serum of rats rises with age. The viscosity of the serum of rachitic rats is not essentially altered from the

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TABLE II.
Normal Young Rats of 70 to 90 Gm.

Serum.		
Viscosity.	Refractivity.	Albumin.
	<i>Pulfrich units</i>	<i>per cent</i>
1.490	51.54	82.60
1.530	52.90	78.50
1.476	51.50	85.00
1.556	54.70	81.15
1.460	49.90	81.30
1.580	55.50	78.50
1.520	50.80	70.10
1.488	50.20	76.80
1.505	52.116	81.07
1.450	51.50	87.27
1.490	50.15	81.00
1.524	53.60	82.83

TABLE III.
Rachitic Rats (80 to 150 gm.) Treated with Cod Liver Oil, Showing Beginnin Healing.

Serum.		
Viscosity.	Refractivity.	Albumin.
	<i>Pulfrich units</i>	<i>per cent</i>
1.699	58.90	74.17
1.575	57.10	86.80
1.631	58.50	81.24
1.645	59.00	81.07
1.654	58.90	78.75
1.601	60.46	91.50
1.651	59.675	81.80
1.681	65.00	80.60
1.709	61.62	79.00
1.660	63.42	92.30
1.612	59.62	86.30
1.641	59.97	84.70
1.644	58.75	79.50
1.660	60.81	83.90
1.587	60.53	95.00
1.615	58.67	84.29
1.657	56.87	69.50
1.655	62.24	89.25
1.615	59.02	88.20

TABLE IV.
Rachitic Rats Fed on Mixture No. 3143.

Serum.		
Viscosity.	Refractivity.	Albumin.
	<i>Pulfrich units</i>	<i>per cent</i>
1.691	63.25	88.93
1.604	61.20	94.40
1.670	61.15	84.00
1.616	59.30	86.07
1.607	55.77	75.50
1.660	59.175	78.50
1.604	59.05	87.70
1.608	60.20	90.60
1.608	59.575	85.50
1.509	56.83	95.10
1.607	60.52	82.50
1.679	60.44	80.00
1.632	61.02	89.40
1.620	58.35	82.60
1.634	60.04	85.60
1.588	60.95	96.05

TABLE V.
Rachitic Rats (80 to 150 gm.) Treated with Cod Liver Oil, Showing Advanced Healing.

Serum.		
Viscosity.	Refractivity.	Albumin.
	<i>Pulfrich units</i>	<i>per cent</i>
1.735	63.95	83.34
1.763	60.58	66.81
1.714	58.975	68.19
1.863	63.550	64.10
1.687	59.225	73.86
1.610	55.75	73.64
1.671	59.77	70.38
1.774	60.60	65.00
1.701	62.625	83.93
1.664	57.10	69.45
1.709	58.35	66.30
1.814	62.87	66.60

normal. The viscosity of the serum of rats healed by cod liver oil is *increased*.

2. There is no significant alteration in the refractive value of the serum of rachitic rats, although they show an average slight diminution below the normal level. The refractivity increases in rats with the age of the animal.

3. In spite of the low serum viscosity of the young animals the albumin content is the same as in the serum of adult animals. The figures for the individual vary over a range of 70 to 87 per cent, giving an average of 81 per cent albumin.

Rachitic rats have a somewhat increased albumin content of 86.4 per cent (ranging in different rats between 75.5 and 96.05 per cent), but only in their average figure and in the broader variation scale.

There is a distinct *decrease in the albumin content* of the serum of rachitic rats which are undergoing treatment with cod liver oil. In the serum of rats showing *advanced healing* on histological examination (a line test of +++) the albumin percentage is below 75 per cent in general. The individual values range mostly between 64 and 74 per cent.

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THE FERMENTATION OF PENTOSES BY *BACILLUS GRANULOBACTER PECTINOVORUM*.*

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In his papers on the fermentation of various sugars, polysaccharides and sugar alcohols, Speakman and his associates (1) have observed numerous differences in the rate of fermentation and in the ratios of the several acids formed. On the basis of these relations he has classified the carbohydrates into two groups, those which ferment normally and those which do not. The normal fermentation is illustrated by glucose and starch in which the acidity reaches a maximum and then falls rapidly to a low level from which it rises slowly toward the end of the fermentation. The abnormal fermentations show little or no break in the acidity. In maize fermentation this break in acidity is associated with a rapid production of neutral bodies, acetone, and butyl alcohol.

In the paper already referred to, Speakman placed xylose and arabinose in the group of carbohydrates producing abnormal fermentations. High acidities with little or no break in the curve were found with both pentoses. Unfortunately, no quantitative data on solvent production are given in his papers so it is impossible to determine to what extent the high acidities interfered with this phase of the process.

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EXPERIMENTAL.

Yeast Water Media.—Starch-free yeast was stirred with ten times its weight of tap water until a smooth suspension was obtained and steamed for 1 to 2 hours, during which time the flask was shaken several times. The flask was then plugged with cotton and sterilized for 2 hours. If the yeast is free from starch the suspension clears rapidly and after 24 hours a clear, light brown liquid can be siphoned off. From 20 to 40 liters were prepared at one time and used as needed. The sugar to be fermented was added to the clear yeast water, the solution was sterilized and then inoculated.

TABLE I.

Production of the Solvents, Acetone and Butyl Alcohol, from Xylose and Glucose.

Yeast water medium containing 2 per cent of sugar.

No.	Sugar fermented.	Solvents per liter of culture.	Sugar recovered in solvents.
		gm.	per cent
1	Control.	Trace.	
2	"	0.60	
3	Xylose.	4.88	21.4
4	"	5.00	22.0
5	Glucose.	5.60	25.0
6	"	5.10	27.5
7	"	5.30	28.5

Peptone-Salt Media.—The composition of this media was that used by Robinson (1). The sugars were added to the peptone-salt mixture in the quantity desired and the whole was sterilized. Strips of filter paper as recommended by Robinson were placed in the flasks in most of the experiments.

Methods of Analysis.—Solvents were determined by neutralizing and distilling the fermented culture and calculating the percentage of solvents from the specific gravity of the distillate. Acetone in the distillate was determined by Goodwin's modification (2) of Messinger's method.

Carbon dioxide was determined by absorption in potassium hydroxide and analyzed by Van Slyke's (3) gasometric method. Volatile and non-volatile acids were determined by distillation and ether extraction, respectively (4).

Experiment 1.—This was a preliminary experiment in which the fermentability of xylose and glucose under our methods of manipulation was tested. A series of 500 cc. Erlenmeyer flasks, each containing 300 cc. of yeast water and 6 gm. of the sugar, was set up and inoculated with 10 cc. of a vigorous culture which had been grown for 24 hours in 6 per cent corn mash. Evolution of gas began in 12 hours and continued for about 48 hours. The flasks were allowed to stand 96 hours and then analyzed.

The acidity of the xylose culture was rather high at the end of the fermentation; 4.0 cc. of 0.1 N acid per 10 cc. of culture. The yield of solvents is given in Table I and shows a good production of solvents from

TABLE II.

Comparison of the Rate of Fermentation of Glucose and Xylose.

Robinson's peptone-salt media.

Time after inoculation.	Glucose.		Xylose.	
	Titrateable acid.*	Sugar in culture.	Titrateable acid.*	Sugar in culture.
<i>hrs.</i>		<i>per cent</i>		<i>per cent</i>
0	1.2	2.0	1.0	2.0
16	3.4	1.2	3.3	1.4
23	4.1	1.0	4.2	1.2
29	4.2	0.1	4.6	0.7
42	3.6	0.0	5.2	0.4
52	2.9	0.0	5.0	
66	3.2	0.0	5.0	0.2
Solvents in 1,000 cc. culture, gm.....		6.30		4.74
Acetone in 1,000 cc. culture, gm.....		1.58		1.29
Acetone in solvents, <i>per cent</i>		25.00		27.20

* Cc. of 0.1 N acid in 10 cc. of culture.

xylose, although not equal to that obtained from glucose. The yields compare favorably with those obtained from corn mash, which is probably one of the best media for the growth of this microorganism.

Experiment 2.—The comparative rate of fermentation of glucose and xylose was tested in this experiment. The results are given in Table II and show that glucose fermented with greater rapidity than xylose. The glucose fermentation was a particularly rapid one, while the xylose fermentation proceeded at about the usual rate. All the glucose and 90 per cent of the xylose were fermented at the end of 66 hours. The most rapid fermentation of xylose reported by Robinson was 52 per cent in 72 hours.

Only a slight break in the acidity occurred in the case of xylose while the glucose acidity showed a decided break. A definite break in the acidity in xylose fermentations is not uncommon, however. In the course of a dozen fermentations we have observed a decrease in titratable acid ranging from 0.5 to 1.3 cc. of 0.1 N acid per 10 cc. of culture. In consequence of the higher residual acidity a smaller yield of solvents was obtained from xylose than from glucose. The acetone-butyl alcohol ratio was about the same in both cases. The destruction of sugar and production of solvents do not agree with the statement by Speakman that abnormal fermentations (xylose, mannitol, etc.) are characterized by a low consumption of carbohydrates and a poor yield of solvents.

Balance between the Sugar Fermented and the Products Formed.

Experiment 3.—In this experiment the total products with the exception of hydrogen were determined and added together to balance against the sugar destroyed. Residual volatile acid was determined by steam distillation which was followed by ether extraction of the residue for the determination of non-volatile acid.

Some assumptions were made in calculating the figures in Table III. The difference between total solvents and acetone is called butyl alcohol, although from 10 to 20 per cent of this weight consists of ethyl alcohol and other minor products.

In calculating the volatile acid an average molecular weight of 70 has been taken. Calculations from Speakman's Duclaux analyses give an average molecular weight of the volatile acids varying from 65 for glucose to 80 for mannitol with intermediate values for xylose and arabinose.

The non-volatile acid is calculated as lactic acid, although no conclusive evidence has as yet been adduced for the existence of lactic acid in a *granulobacter* fermentation.

In all cases a slightly larger weight of products was obtained than sugar destroyed. The excess originates, probably, from yeast water. This contains about 1 gm. of carbohydrate and about 4 gm. of crude protein per liter.

The volatile acid in a fermented culture is usually less for glucose than for either of the pentoses. Titration curves show that the break in acidity is generally less marked for these compounds than for glucose.

TABLE III.

Total Fermentation Products Obtained from Pentoses and Glucose.

Yeast water media. Calculated for 1,000 cc. of culture.

Compound fermented.	Weight of compound fermented.	Solvents.		Volatile acid.*	Non-volatile acid as lactic.	CO ₂	Total products.
		Butyl alcoh.*	Acetone.				
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Yeast water.	1.1†	0.26	0.13	0.02	0.14	0.38	0.93
" "	1.2‡	0.28	0.14	0.02	0.23	0.53	1.20
Xylose.	19.1	4.61	2.19	2.37	0.77	11.25	21.19
"		3.27	1.93	2.03			
"	17.6	4.60	0.93	3.85			
Arabinose.	19.4	3.43	2.77	3.56	0.47	11.30	21.53
"	19.2	3.61	2.59	3.65	0.67	11.63	22.15
Glucose.	19.1	4.64	1.76	2.12	1.17	11.71	21.40
"	19.0	4.21	2.19	1.86	0.74	10.83	19.83

* Total solvents minus acetone.

† Average molecular weight of 70 used in calculations.

‡ Unfermented sugar.

SUMMARY.

The fermentation of xylose and arabinose by *Bacillus granulobacter pectinovorum* results in the same products and in essentially the same quantities as from glucose. Slightly less solvents and slightly more volatile acids are produced from the pentoses.

The rate of fermentation is somewhat slower for the pentoses than for glucose; but practically all the sugar is destroyed in 72 hours.

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HYDROGEN ION CONCENTRATION IN THE HUMAN DUODENUM.

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We have recently had the unique opportunity of measuring *in vivo*, so the speak, the reaction of the duodenum of a man in whom a duodenal fistula had been established.

The measurements were made electrometrically by means of a Leeds and Northrup potentiometer No. 7665 reading to 0.001 volts, an Eppley standard cell, a D'Arsonval portable galvanometer, a calomel cell of standard type, and a special hydrogen electrode which was designed to fit the peculiar experimental conditions existing in this case. The structural details of this instrument are shown in Fig. 1.

The electrode differs from the ordinary Hildebrand type in two respects, a screen of fine mesh silver wire was placed around the platinum tip to prevent contamination by contact with large particles of foodstuffs or other materials, and a glass tube of 2 mm. bore was attached as illustrated to furnish connection with the calomel cell through an intermediate vessel. The length of the electrode was 15 cm. and its greatest diameter was 1 cm. A piece of No. 22 platinum wire was used in making the tip, and was hammered into a thin strip 1.5 mm. wide and 8 mm. long. This electrode could be inserted through the fistula and allowed to lie in the lumen of the intestine. The average depth to which the electrode was inserted in the intestine in our experiments was 6 cm. and on one occasion a depth of 9 cm. was attained. Hydrogen was generated in a Kipp apparatus and was supplied to the electrode under a hydrostatic pressure of about 15 cm. Normal KCl solution was used in the calomel cell and connecting vessel and the apparatus was thoroughly tested in

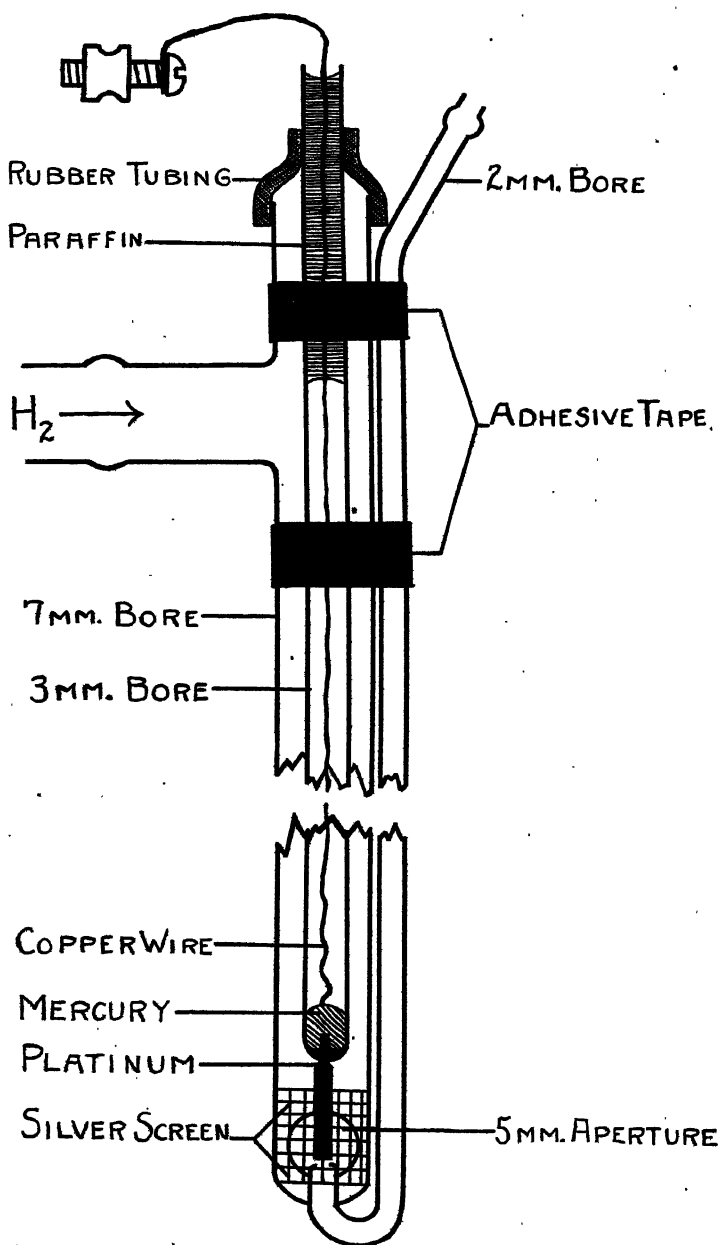


FIG. 1. Modified type of Hildebrand hydrogen electrode.

the laboratory by determining the pH values of a series of buffers which had been previously standardized electrometrically. As a check on the apparatus, tests on samples of these same standard buffers were made at the patient's bedside just before insertion of the electrode in the intestine and immediately after its removal. In each of the four experiments in which this check was applied it was found that the maximum deviation from the known pH of the buffer was -0.12 pH and the average deviation ± 0.05 pH.

As the fluid under examination was believed to be flowing past the electrode more or less continuously (an assistant being kept busy sponging up the overflow from the fistula throughout each experiment), we have assumed it reasonable to suppose that the original CO_2 content of the material was not changed appreciably by the use of a bubbling type of hydrogen electrode.

Our subject, a colored laborer, 23 years of age, was an inmate of the New Orleans Charity Hospital, to which institution he had been admitted on December 21, 1923, suffering from an injury caused by a pistol bullet which had entered the right side immediately below the costal border. On operation, which was performed by Dr. E. L. Irwin a few hours after admission, repair was made of three perforations of the colon, and, as it was found that the projectile had traversed the liver and had made exit immediately posterior to the gall bladder, perforating this organ at its attachment to the liver, it was deemed advisable to perform a cholecystostomy.

After the above operation the patient made satisfactory progress until January 6, 1924, when during a fit of coughing the suture line gave way, and the intestines were found out of the abdominal cavity. In an emergency operation the abdominal wound was again closed. Progress now continued satisfactory until February 22, 1924, when it was noted that a fistulous opening had appeared in the upper third of the abdominal wound. On exploration of the fistulous tract it was found that just within the abdominal wall there existed a fistulous pocket which extended over the entire upper right quadrant, and which was sealed off from the general abdominal cavity by adhesions.

Further exploration gave definite evidence of the duodenal origin of the fistula.

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Experiment 1.

Record of results obtained in an investigation of the pH of intestinal contents by direct electrometric determination in a duodenal fistula.

April 3, 1924.

Time.	Reading in volts.	pH	Remarks.
a.m.	0.735	7.66	These figures obtained in checking electrode at patient's bedside with a standard buffer of pH 7.74.
9.50	0.678	6.69	
	0.679	6.71	
	0.679	6.71	
	0.678	6.69	
	0.690	6.89	Rapid flow of juice.
9.55	0.688	6.86	
	0.689	6.88	
10.00	0.675	6.66	
	0.674	6.64	
	0.676	6.67	Flow continues.
	0.674	6.64	
10.05	0.675	6.66	200 cc. milk given.
	0.674	6.64	
10.06	0.675	6.66	
10.07	0.678	6.69	
10.09	0.672	6.59	
10.09½	0.673	6.62	
10.11	0.680	6.73	
10.13	0.685	6.81	
10.14	0.687	6.84	
10.15	0.688	6.86	
10.16	0.690	6.89	
10.17½	0.691	6.92	
10.20	0.693	6.95	
10.21	0.694	6.97	Flow small.
10.23	0.693	6.95	Adjustment of working current checked against standard cell.
10.24	0.693	6.95	
10.25	0.693	6.95	
10.26	0.694	6.97	
10.28	0.693	6.95	
10.30	0.694	6.97	Very small flow.
10.31	0.694	6.97	
10.32½	0.696	7.01	
10.35	0.698	7.03	
10.37	0.699	7.05	200 cc. milk given.

Time.	Reading in volts.	pH	Remarks.
a.m.			
10.39	0.700	7.06	
10.40	0.703	7.12	
10.41	0.705	7.14	
10.42	0.706	7.15	
10.45	0.715	7.33	
10.46	0.713	7.28	First curd appears.
10.47	0.713	7.28	
10.48	0.706	7.15	
10.49	0.703	7.12	
10.50	0.697	6.99	
10.51	0.697	6.99	
10.51½	0.695	6.98	
10.52	0.692	6.94	
10.54	0.693	6.95	
10.54½	0.692	6.94	
10.55	0.689	6.88	
10.57	0.689	6.88	
10.59	0.694	6.97	
10.59½	0.688	6.86	
11.01	0.692	6.94	
11.02	0.695	6.98	
11.03	0.698	7.03	
11.04	0.701	7.08	
11.09	0.737	7.69	This reading obtained on standard buffer solution immediately after removal of electrode from the intestine. True pH 7.74.

The patient had had no food for 15 hours previous to the carrying out of this experiment.

After the third operation the patient continued to make satisfactory progress, the duodenal fistula still persisted however, and continued to discharge bile and intestinal contents. Excoriation of the skin in the region of the wound was prevented by the liberal use of gauze dressings which were changed at frequent intervals by the patient himself who gained rapidly in weight and strength, and who apparently, as judged by the

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Experiment 2.

April 6, 1924.

Time.	Reading in volts.	pH	Remarks.
<i>a.m.</i>			
	0.686	6.83	Check on electrode at patient's bedside with standard buffer of pH 6.80. Patient has had no food for 16 hrs.
9.46	0.686	6.83	
9.50	0.693	6.95	
9.52½	0.699	7.05	Practically no flow of secretion while the above were being taken.
9.55	0.703	7.12	
9.57			Adjustment of working current checked against standard cell.
9.58	0.705	7.14	
10.00	0.708	7.20	
10.02	0.711	7.26	
10.03½	0.713	7.28	
10.06			Adjustment of working current checked against standard cell.
10.07	0.726	7.50	
10.08½	0.727	7.52	
10.09½	0.731	7.59	
10.10			Adjustment of working current checked against standard cell.
10.11	0.733	7.62	
10.12	0.732	7.60	
10.13½	0.735	7.65	
10.16	0.704	7.13	Sudden secretion of juice.
10.19	0.700	7.06	
10.23	0.711	7.26	Sudden large flow of juice.
10.25	0.705	7.14	
10.27			200 cc. 30 per cent cream given.
10.28	0.729	7.55	
10.31	0.728	7.53	
10.33	0.743	7.79	
10.35	0.750	7.91	Sudden flow of juice.
10.37	0.675	6.66	
10.39	0.678	6.69	
10.41	0.683	6.78	
10.43	0.685	6.81	
10.48	0.691	6.92	Adjustment of working current checked against standard cell.
10.50	0.712	7.27	

Time	Reading in volts.	pH	Remarks.
<i>a.m.</i>			
10.53	0.716	7.34	Secretion becomes very plentiful.
10.55	0.704	7.13	
10.57	0.681	6.75	200 cc. 30 per cent cream given.
10.58	0.698	7.05	Curds appear in secretion.
11.01	0.715	7.32	Secretion continues plentiful.
11.03	0.703	7.12	
11.09	0.726	7.50	
11.12			Adjustment of working current checked against standard cell.
11.16	0.699	7.05	
11.20	0.715	7.33	
11.22	0.687	6.84	
11.26	0.697	6.99	
11.30	0.695	6.98	
11.37	0.678	6.69	
11.44	0.692	6.94	
11.49	0.689	6.88	Adjustment of working current checked against standard cell.
11.51	0.679	6.71	
11.54	0.678	6.69	Final check on standard buffer pH 6.81 immediately after removal of the tube from the patient.

amount and quality of the food consumed, was possessed of unusually good digestive powers.¹

When we began work on this case the fistula was so large that the electrode could easily be slipped into the intestine, in which position it could lie for several hours, apparently without the least discomfort to the patient, who, although somewhat apprehensive at the beginning of the first experiment soon became satisfied that no pain would be inflicted, and would readily eat and drink with evident pleasure and frequently sleep, during the course of an experiment.

¹ Quantitative determinations of the concentration of amylase, lipase, and protease carried out by the method of McClure, Wetmore, and Reynolds (1) on samples of the fistula fluid collected at intervals during these experiments invariably gave results indicative of the fact that normal amounts of these enzymes were present in the secretion.

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Experiment 3.

April 8, 1924.

Time.	Reading in volts.	pH	Remarks.
<i>a.m.</i>			
9.15	0.684	6.80	Check on electrode at patient's bedside with standard buffer of pH 6.81.
9.26	0.685	6.81	Active secretion containing much bile.
9.27	0.687	6.84	
9.29	0.681	6.75	
9.31	0.682	6.76	Flow less active.
9.35	0.699	7.05	
9.39	0.715	7.33	Adjustment of working current checked against standard cell.
9.43	0.722	7.44	Very little secretion.
9.46	0.732	7.60	Secretion almost stopped.
9.48	0.731	7.59	Fed 8 egg whites and 1 yolk, boiled 2 min., + a small amount of NaCl.
9.49	0.722	7.44	
9.56	0.698	7.03	Finished feeding.
9.57			Secretion almost stopped. Adjustment of working current checked against standard cell.
10.01	0.690	6.89	
10.04	0.693	6.95	Practically no secretion.
10.07	0.694	6.97	
10.11	0.707	7.17	
10.14	0.718	7.37	
10.18	0.731*	7.59	Adjustment of working current checked against standard cell.
10.20	0.722	7.44	Secretion becoming active.
10.25	0.734	7.63	Albumin appears in fistula.
10.30	0.735	7.65	
10.35	0.715	7.33	Large flow of secretion.
10.40	0.694	6.97	
10.42	0.692	6.94	
10.44	0.683	6.78	Very copious flow.
10.48	0.631	5.90	Moderate flow, very light colored, almost no bile, big clot of albumin.
10.50	0.662	6.42	
10.50½	0.668	6.52	More bile coming through.
10.53	0.672	6.59	Flow almost stopped.

Time.	Reading in volts.	pH	Remarks.
<i>a.m.</i>			
10.55	0.694	6.97	Very marked flow.
10.59	0.695	6.98	
11.04	0.672	6.59	
11.06	0.669	6.54	Almost no secretion.
11.08	0.667	6.51	
11.13	0.664	6.45	Patient asleep.
11.18	0.672	6.59	Secretion clear and free from clots.
11.22	0.678	6.69	
11.23	0.663	6.43	Patient awake. Adjustment of working current checked against standard cell.
11.26	0.662	6.42	
11.30	0.681	6.75	Secretion limited in amount. This reading obtained on standard buffer pH 6.81 immediately after removal of electrode from patient's intestine. Fasting period 16 hrs.

Little comment is necessary in connection with the results obtained during the above series of observations.

The following tabulation gives the average and extreme variations of pH found in each experiment and in the entire series; and as will be noted the average pH for each experiment is approximately the same, a finding which would seem to indicate that the reaction is unaffected by the nature of the food eaten.

		pH
Experiment 1. (Milk.)	Minimum.....	6.59
	Maximum.....	7.33
	Average.....	6.91
Experiment 2. (Cream.)	Minimum.....	6.66
	Maximum.....	7.91
	Average.....	7.17
Experiment 3. (Egg whites.)	Minimum.....	5.90
	Maximum.....	7.65
	Average.....	6.95
Experiment 4. (Starch.)	Minimum.....	6.19
	Maximum.....	8.23
	Average.....	7.07
Extreme variations of entire set of experi- ments.	Minimum.....	5.90
	Maximum.....	8.23
	Average (computed from 182 readings)	7.02

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Experiment 4.

April 17, 1924.

Time.	Reading in volts.	pH	Remarks.
<i>a.m.</i>			
	0.736	7.67	Check on electrode at patient's be- with standard buffer of pH 7.74.
9.45	0.676	6.67	Almost no secretion.
9.49	0.682	6.76	No bile present.
9.50	0.679	6.71	Adjustment of working current che against standard cell.
9.53	0.682	6.76	
9.56	0.685	6.81	
9.57½	0.678	6.69	
10.00	0.675	6.66	
10.01	0.681	6.75	Adjustment of working current che against standard cell.
10.03	0.684	6.79	No bile, very little secretion.
10.05	0.696	7.01	Sudden flow of secretion with bile.
10.11	0.686	6.83	200 cc. water given.
10.14	0.707	7.17	Started eating 10 crackers (Arrowroot
10.17	0.720	7.40	
10.18			10 more crackers. Adjustment of wor current checked against standard ce
10.18½	0.705	7.14	Flow almost stopped.
10.21	0.691	6.92	
10.23			10 more crackers, flow starts again, no
10.25	0.671	6.58	
10.27	0.705	7.14	Moderate flow, some bile.
10.29	0.768	8.23	
10.31	0.742	7.78	Sudden outpouring of secretion, 4 crackers.
10.33	0.722	7.44	Crackers appear in secretion.
10.35			Finished crackers, drank 200 cc. water
10.37½	0.729	7.56	
10.38½	0.753	7.96	
10.39			Sudden flow of fluid.
10.41	0.705	7.14	Patient asleep.
10.46	0.758	8.05	Almost no secretion.
10.50	0.739	7.74	
10.51½	0.723	7.45	Marked flow of secretion.
10.54			" " " "
10.54½	0.698	7.03	
10.58	0.701	7.08	Almost no secretion.
11.05	0.648	6.19	Sudden large flow.
11.09	0.689	6.88	Patient just awakes, large amount of c ers in secretion.

* Total weight of crackers 153 gm., which were calculated to co
110 gm. of arrowroot-starch.

Time.	Reading in volts.	pH	Remarks.
<i>a.m.</i>			
11.14	0.702	7.10	
11.17	0.726	7.50	Large flow.
11.18	0.712	7.27	Adjustment of working current checked against standard cell.
11.19	0.691	6.92	Large flow continues.
11.22	0.715	7.32	Flow moderate.
11.24	0.671	6.58	Large flow starting.
11.27	0.689	6.88	No flow..
11.30	0.697	6.99	Started to eat apple pie.
11.33			Finished pie.
11.33½	0.676	6.67	No secretion.
11.36	0.681	6.75	Almost no secretion.
11.38	0.686	6.83	
11.47½	0.678	6.69	Very small amount of secretion.
11.50	0.738	7.71	This reading on standard buffer of pH 7.74.

Patient had no food for 15 hours previous to the taking of these figures.

Our average results are on the whole in accord with the views now held regarding the reaction of the duodenal contents in man, but in no case do our minimal values reach those recorded by some previous investigators. McClendon (2) gives pH 7.7 as the hydrogen ion concentration of the adult duodenum. Long and Fenger (3) state that as a result of their observation on the duodenal secretions removed from normal men by means of a Rehfuß tube shortly after the subjects had been fed an ordinary meal, they believe the reaction of the duodenum may be either acid or alkaline; results as low as pH 2.27 and as high as 7.81 having been obtained. Myers and McClendon (4) in a series of experiments made on a single normal subject who was fed an ordinary diet reported values ranging from pH 3.2 to 7.82 in specimens removed by tube 3 to 4 hours after meals.

Okada and Arai (5) who determined the reaction in the duodenal secretions of fourteen hospital patients who were suffering from a variety of pathological conditions obtained figures varying from pH 4.80 to 7.97.

The most recent publication on the subject is that of McClure, Montague, and Campbell (6) who have determined the reaction of the duodenal contents removed from normal men after the

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ingestion of meals consisting of practically pure fat, carbohydrate, or protein, taken in the form of olive oil, arrowroot-starch, or edestin, or mixtures of these. As a result of their observations these investigators state that the duodenal contents were acid after the ingestion of protein and mixtures of food substances, and alkaline after the ingestion of fat and carbohydrate foods. The actual figures given vary from pH 3.172 to 8.102.

As far as we know, our experiments described above are the only ones on record in which the reaction of the duodenal content has been measured directly by the electrometric method, *viz.* without the use of a duodenal tube, and, as the highest acidity recorded in this series is only pH 5.90, the hypothesis suggests itself that the presence of the tube in the pylorus may cause the sphincter to remain open longer than when no foreign body is present, thereby allowing larger portions of gastric contents to enter the duodenum than are usually passed under ordinary conditions, and thus producing greater fluctuations in the reaction of the duodenum than those noted in our case.

While it is perhaps outside of the scope of this investigation we have noted with interest that the time of appearance of fragments of food in the liquid exuding from the fistulous opening bore the same relation to the type of food taken as has been described by Cannon (7); *viz.*, the carbohydrate meal passed into the intestine at a rate which was distinctly more rapid (19 minutes) than that taken by the cream, egg white, or milk which latter substance appeared at 31, 37, and 41 minutes, respectively. It should be noted that the milk was given during the first experiment at a time when the patient was distinctly apprehensive, so that a psychic inhibition of the pyloric sphincter may be suspected in this case.

SUMMARY.

Electrometric determinations of the hydrogen ion concentration of the human duodenum made by insertion of an electrode through a fistulous opening gave a maximum value of pH 8.23 and a minimum of pH 5.90, while the average calculated from 182 readings was pH 7.02. No definite difference in reaction was noted in observations made after the ingestion of meals consisting largely of fat, carbohydrate, or protein.

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SOME NITROGENOUS CONSTITUENTS OF THE JUICE OF THE ALFALFA PLANT.

I. THE AMIDE AND AMINO ACID NITROGEN.*

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INTRODUCTION.

In the extensive study of the chemical composition of the alfalfa plant which has been carried on in this laboratory for the past 2 years, much attention has been given to the nature of the nitrogenous substances which occur dissolved in the plant juice (1, 2). Particular attention is devoted in this paper to the amide and amino acid nitrogen. In a subsequent paper the results of a study of the basic substances will be given.

The fresh juice was treated with 53 per cent by weight of 96 per cent alcohol and the protein and inorganic salts were filtered off.¹ The filtrate was then concentrated *in vacuo* and alcohol added until a further precipitate began to separate. The fluid thus prepared contains about 20 per cent of solids, gives no protein reaction, and is referred to as the "alfalfa filtrate." This filtrate contains red-brown pigments which occur free and in combinations which are not themselves highly colored; but which readily yield an intense color on mild hydrolysis. Pigments, or complexes readily yielding them, are encountered in every fraction, and greatly increase the difficulties of isolation of single chemical individuals.

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The writer wishes to express his thanks to Dr. Thomas B. Osborne for his interest in the work and also for much helpful advice and criticism.

¹ These precipitates have already been studied in some detail; see Osborne, Wakeman, and Leavenworth (1, 2).

The essential features of the fractionation are presented in the following scheme.

Scheme of Fractionation.

Step 1. Precipitation of the "alfalfa filtrate" with normal lead acetate.

Step 2. Precipitation of the filtrate from Step 1 with mercuric acetate, sodium carbonate, and alcohol (Neuberg and Kerb's reagent (3)).

Step 3. Precipitation, with phosphotungstic acid, of the solution obtained by decomposing the precipitate of Step 2.

Step 4. Fractional crystallization of amides and amino acids from the filtrate from the phosphotungstic acid precipitate.

Step 5. Separation of the solution obtained by decomposing the precipitate of Step 3 into purine, arginine, and lysine fractions, by precipitations with silver sulfate and silver sulfate together with baryta, respectively.

Step 6. Precipitation, with mercuric chloride in acid solution, of betaines from the filtrate obtained in Step 2.

The above scheme has been designed to furnish as much information as possible regarding the groups of nitrogenous constituents of the alfalfa filtrate.

Schulze² and his collaborators have been the most extensive workers in this field in the past, but they have devoted their efforts chiefly to demonstrating the presence of individual substances in a *group of plant species*; we have tried to learn as as much as possible about each of the fractions obtained from a *single plant*.

GENERAL DISCUSSION.

1 liter of the alfalfa filtrate represents the juice from about 6,650 gm. of the fresh plant. The analysis is given in Table I.

Normal lead acetate precipitated 10.7 per cent of the total nitrogen, 15.2 per cent of the amino nitrogen, and 14.2 per cent of the organic solids of the alfalfa filtrate. In regard to the nature of the constituents of this precipitate we are not yet prepared to make any definite statement.

Neuberg's reagent precipitates amino acids and certain basic substances. The latter can be removed by means of phosphotungstic acid. Table II shows the forms of nitrogen in the precipitate obtained with Neuberg's reagent.

² See for example, Schulze, E., *Z. physiol. Chem.*, 1898, xxiv, 18.

Table III shows the forms of nitrogen in the basic substances precipitated by Neuberg's reagent.

Table IV gives the forms of nitrogen in the filtrate from the bases.

TABLE I.

	Amount.	Total nitrogen.
	gm.	per cent
Total nitrogen.....	10.06	
Ammonia "	0.4066	4.04
Amide* "	0.8344	8.29
Amino "	3.67	36.5
Other "	5.15	51.24
Solids	175.0	
Ash.....	28.2	

* Amide nitrogen was determined by boiling for 4 hours with 4 per cent HCl, adding excess magnesia, and distilling the ammonia into standard acid. The free ammonia, determined in another aliquot, was subtracted. This is essentially Sachsse's method (Sachsse, R., *J. prakt. Chem.*, 1873, vi, 118).

TABLE II.

Precipitated by Neuberg's Reagent.

	From 1,000 cc. alfalfa filtrate.	Of each form of nitrogen in 1,000 cc. alfalfa filtrate.
	gm.	per cent
Total nitrogen.....	5.81	57.8
Ammonia "	0.054	13.3
Amide "	0.731	87.6
Amino "	2.58	70.0
Ash-free solids	44.45	25.4

Solids are 13.01 per cent nitrogen.

Some nitrogen was unaccounted for in the two fractions of the mercury precipitate obtained by means of phosphotungstic acid. A similar loss of nitrogen was encountered by Osborne, Wakeman, and Leavenworth when dealing with their phosphotungstic acid precipitates from alfalfa juice.³

³ Osborne, Wakeman, and Leavenworth (1), p. 421.

650 Nitrogenous Constituents of Alfalfa. I

Amides are regarded as important constituents of plant juices. The filtrate from the phosphotungstic acid precipitate contained 0.561 gm. of amide nitrogen, representing 67 per cent of the possible maximum. From this solution 3.193 gm. of asparagine, containing 0.298 gm. of amide nitrogen, were isolated by fractional crystallization. Thus only somewhat over one-half of the amide nitrogen in it was accounted for as crystalline asparagine. Some amide

TABLE III.

	From 1,000 cc. alfalfa filtrate.	Of each form of nitrogen in 1,000 cc. alfalfa filtrate.
	<i>gm.</i>	<i>per cent</i>
Total nitrogen.....	1.951	19.4
Ammonia ".....	0.043	10.6
Amide ".....	0.095	11.4
Amino ".....	0.502	13.7
Ash-free solids.....	12.34	7.06

Solids are 15.8 per cent nitrogen.

TABLE IV.

	From 1,000 cc. alfalfa filtrate.	Of each form of nitrogen in 1,000 cc. alfalfa filtrate.
	<i>gm.</i>	<i>per cent</i>
Total nitrogen.....	2.65	26.4
Ammonia ".....	0.008	1.9
Amide ".....	0.561	67.3
Amino ".....	1.66	45.4
Ash-free solids.....	23.28	13.3

Solids are 11.4 per cent nitrogen.

other than asparagine is doubtless present, but we have as yet failed to isolate it.

Only 35.7 per cent of the amide nitrogen in 1 liter of alfalfa filtrate was actually accounted for as amide nitrogen of a definite amide; namely, asparagine. This is 2.96 per cent of the total nitrogen of the alfalfa filtrate. Asparagine, therefore, accounts for 5.82 per cent of the nitrogen and 1.82 per cent of the organic solids of the alfalfa filtrate.

Schulze (4) likewise failed to obtain as asparagine any large proportion of the amide nitrogen of the juice of mature fresh plants.

Butkewitsch (5) obtained from *Vicia faba* little more than 30 per cent of the asparagine as calculated from the amide nitrogen, but larger proportions from plants in which the asparagine content had been greatly increased by water culture in the dark.

Glutamine has been found in many plants, especially in seedlings (6), by Schulze. No other amides than asparagine and glutamine have yet been isolated from plants (7), but Schulze (8) has pointed out the probability that such must occur. We have found no indication of the presence of glutamine in alfalfa.

The amino nitrogen of the alfalfa filtrate which belongs to free α -amino acids, or to simple peptides, is largely concentrated in the same fraction as the asparagine. Deducting the amino nitrogen of the 3.193 gm. of asparagine from the 1.66 gm. of amino nitrogen in this solution, leaves 1.362 gm. of amino nitrogen, which is only 13.6 per cent of the total nitrogen of the alfalfa filtrate; a relatively small proportion.

Tyrosine in a yield of 0.224 gm., containing 0.0175 gm. of nitrogen, was isolated in pure form from 1 liter of alfalfa filtrate. This represents less than 0.2 per cent of the total nitrogen. The complete investigation of the amino acid make-up of this fraction will have to await the application of the methods of protein analysis to much larger quantities of material than we had at our disposal.

The presence of peptides in this solution was demonstrated indirectly by the hydrolysis of a small fraction obtained as a copper salt. Before hydrolysis this fraction contained 69.5 per cent of its nitrogen as amino nitrogen, while afterwards the ratio was 89 per cent. The mother liquor, from which as much material as possible had been removed by direct crystallization and by crystallization of copper salts, likewise contained peptides since, when allowance was made for free ammonia and amide nitrogen, the ratio of amino to total nitrogen was increased from 63.5 to 77.6 per cent by hydrolysis.

EXPERIMENTAL PART.

1 liter of alfalfa filtrate, containing about 30 per cent of alcohol by volume, was treated with approximately 120 gm. of normal lead acetate in 20 per cent aqueous solution. The reaction of the juice before precipitation was between pH 4 and 5 and was practically unchanged by the addi-

tion of the reagent. The precipitate was washed with water, decomposed with hydrogen sulfide, and the solution and washings were concentrated to 1,000 cc. This contained 24.89 gm. of organic solids and 1.08 gm. of nitrogen, of which 0.038 gm. was free ammonia and 0.559 gm. amino nitrogen. The high proportion of amino to total nitrogen and the fact that over 15 per cent of the amino nitrogen of the juice appears in this precipitate are of interest.

The filtrate from the normal lead acetate precipitate was treated with 20 per cent sodium carbonate solution until the reaction was alkaline to litmus, but still acid to phenolphthalein. The precipitate contained only 0.18 gm. of nitrogen and was discarded. The filtrate was then made neutral to litmus with acetic acid, concentrated to about 2 liters, freed from a trace of lead with hydrogen sulfide, and, after removal of an aliquot for analysis, treated alternately with 20 per cent sodium carbonate and 20 per cent mercuric acetate until further additions of either reagent gave an orange precipitate. This required 180 gm. of mercuric acetate. An equal volume of 96 per cent alcohol was then added and the precipitate centrifuged, washed with 50 per cent alcohol, and decomposed with hydrogen sulfide. The mercuric sulfide was removed and washed and the solution made to 1,000 cc. The filtrate from the mercuric acetate precipitate was acidified with acetic acid, freed from mercury with hydrogen sulfide, and concentrated to 2,000 cc. Analyses of these solutions showed that only small losses of nitrogen had occurred.

To the chilled solution of the substances precipitated by Neuberg's reagent 5 per cent of sulfuric acid was added and 20 per cent phosphotungstic acid solution until precipitation was complete. The precipitate was centrifuged off, washed with 5 per cent phosphotungstic acid wash solution, and thoroughly treated with excess of cold saturated baryta solution. The barium phosphotungstate was then treated with warm baryta and finally digested twice more on a steam bath with baryta solution. Each extract was at once acidified to pH 4 to 5 with sulfuric acid. All were united, filtered from barium sulfate, and concentrated to 1 liter. The examination of this solution will be reported later.

As soon as the phosphotungstic acid precipitate had been removed the filtrate was made slightly alkaline with baryta. Since this solution was strongly acid for not more than 2 hours hydrolysis of amides probably did not occur to any great extent. The barium sulfate and phosphotungstate were then filtered out, excess of baryta was quantitatively removed with sulfuric acid, and the solution made to 1 liter. This will subsequently be referred to as the amino acid solution. The analysis of these two fractions of the substances precipitated by Neuberg's reagent has been given in the earlier part of this paper (pp. 649 and 650).

The amino acid solution was concentrated under diminished pressure to about 40 cc. and allowed to stand in a cold place for 2 days during which time crystals separated. On recrystallizing from water asparagine mixed with tryosine was obtained. These were separated by elutriation and each obtained pure by repeated crystallization. On further concentration the

solution yielded a second crop of impure crystals from which more asparagine and tyrosine were obtained, as well as a crop of ill defined nodules and scales which was removed separately. This crop weighed only 0.27 gm. and was not further investigated. The mother liquor was next treated with sufficient absolute alcohol to precipitate a heavy sirup. On standing several weeks crystals separated which were almost entirely asparagine. The yield of pure asparagine was equivalent to 3.193 gm. from 1 liter of alfalfa filtrate. Dried at 110° this lost 11.98 per cent of water and contained 18.58 per cent of nitrogen. Theory for $C_4H_8O_3N_2 \cdot H_2O$; H_2O , 12.00 per cent, N, 18.66 per cent.

A determination by Sachsse's method gave 10.54 per cent of amide nitrogen. Theory for $C_4H_8O_3N_2$ is 10.61 per cent.

The pure tyrosine obtained was equivalent to 0.224 gm. from 1 liter of alfalfa juice. This contained 7.87 per cent of nitrogen. Theory, 7.74 per cent.

The mother liquor, from which no more asparagine could be crystallized, was boiled with excess of copper hydroxide, filtered while hot, and concentrated to 200 cc. A small precipitate separated on standing which was decomposed with hydrogen sulfide, but pure crystallized asparagine could not be obtained from it. After hydrolysis it was found to contain 0.024 gm. of nitrogen, of which 0.0125 gm. was ammonia. It probably contained asparagine, but the evidence is not conclusive.

On further concentration two successive crops of semicrystalline copper salts were obtained, the first of which on decomposition and fractional crystallization yielded nodules containing 9.4 per cent of nitrogen, of which 86 per cent was amino nitrogen. This material was therefore not asparagine. The second crop on decomposition yielded a sirup which was converted to a brown powder by means of absolute alcohol. This contained 69.5 per cent of amino nitrogen. When boiled with 20 per cent hydrochloric acid for 16 hours the proportion of amino nitrogen rose to 89 per cent. Tyrosine was absent as shown by a negative Millon's test. The powder doubtless consisted of a mixture of amino acids and peptides.

Copper was now removed from the mother liquor, and total and amino nitrogen determined before and after hydrolysis by boiling with 20 per cent hydrochloric acid for 16 hours. The ammonia set free by hydrolysis was also determined. The data are given in Table V.

The amide nitrogen in the solution before hydrolysis was, therefore, 0.159 gm. Assuming that only amides of dibasic amino acids are present the above data indicate that over 14 per cent of the nitrogen in this solution may be in the peptide form.

On account of the notable amount of amide nitrogen in the unhydrolyzed solution a further attempt was made to obtain asparagine from it by following Schulze's method of precipitation with mercuric nitrate. The precipitate, at neutral reaction to litmus, produced by the alternate addition of mercuric nitrate and sodium hydroxide, was decomposed with hydrogen sulfide and concentrated under diminished pressure with frequent additions of ammonia to preserve neutrality to litmus. No asparagine

was obtained, although analyses showed that the mercuric nitrate reagent precipitated 86 per cent of the amide nitrogen.

In view of these failures to increase the yield of asparagine an attempt was next made to obtain aspartic acid after hydrolysis. The solution was concentrated *in vacuo* at low temperature in the presence of excess of calcium hydroxide until free ammonia was removed. The filtered solution was boiled for 16 hours with 20 per cent hydrochloric acid, concentrated *in vacuo*, and the ammonia, set free by the hydrolysis, removed by distillation with excess of calcium hydroxide at low temperature. The concentrated solution containing the calcium salts of the dibasic amino acids was filtered and precipitated by the addition of a large volume of alcohol according to Foreman's (9) method. The precipitate was dissolved in water and calcium removed quantitatively with oxalic acid. The solution was then boiled with excess of copper hydroxide, filtered, and concentrated to small volume. No copper aspartate was obtained. Copper was therefore removed with hydrogen sulfide and the solution concentrated to very small volume and saturated with hydrogen chloride gas. A crystalline substance readily separated in small amount. On recrystallization from dilute

TABLE V.

	Before hydrolysis.	After hydrolysis.
	gm.	gm.
Total nitrogen.....	1.134	1.093
Free ammonia nitrogen.....	0.049	0.208
Humin nitrogen.....		0.041
Amino ".....	0.655	0.723

hydrochloric acid the substance was obtained in long needles and apparently pure. As it melted with decomposition and sublimation at 287-290° it could not have been glutaminic acid hydrochloride (m.p. 197°). Less than 0.1 gm. of this substance was obtained. Its nature will have to await further investigation.

SUMMARY.

The proteins and much inorganic matter dissolved in the juice expressed from the fresh ground alfalfa plant are precipitated by the addition of alcohol to a concentration of 53 per cent by weight. The filtrate is concentrated and preserved by the addition of alcohol. This paper deals with the amide and amino acid nitrogen of this filtrate.

A method of fractionation has been employed which is designed to permit the classification of the substances in the plant juice into groups of similar chemical nature as well as the isolation of individual substances.

Asparagine was isolated to the extent of 1.8 per cent of the organic solids and containing 5.8 per cent of the nitrogen of the alfalfa filtrate, but only about one-third of the amide nitrogen of this filtrate, as determined by Sachsse's method, is thus accounted for. Other amides are probably present, but have not yet been identified.

Free amino acids make up a relatively small proportion of the nitrogen of the alfalfa filtrate since only 13.6 per cent of the total nitrogen of the alfalfa filtrate occurs as amino acid nitrogen in the fraction in which the simple α -amino acids are concentrated.

Tyrosine was isolated in very small amount.

Indirect evidence is given of the presence of a small proportion of polypeptides in the juice of the alfalfa plant.

A paper describing the isolation of certain basic substances from the alfalfa filtrate will shortly appear.

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THE RELATION OF ACIDOSIS AND HYPERGLUCEMIA TO THE EXCRETION OF ACIDS, BASES, AND SUGAR IN URANIUM NEPHRITIS.

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The development of acidosis, hyperglucemia, and glucosuria as well as typical nephritis after the injection of uranium salts has been demonstrated by numerous workers. MacNider (1) has shown that the acidosis associated with this condition may be relieved by the administration of sodium bicarbonate. Karsner, Reimann, and Brooks (2) believe that the glucosuria which occurs soon after the administration of uranium is due to an increased permeability of the kidney. A similar conclusion has been reached by Wallace and Pellini (3) who did not, however, follow the course of the nephritis sufficiently long to observe the hyperglucemia which invariably develops during the later stages of the intoxication. MacNider (1) has pointed out that acetone bodies are formed in uranium nephritis. He regards this as an indication of a general intoxication, resembling somewhat that produced by cyanides. Nuzum and Rothschild (4) have shown recently an increased excretion of organic acids in rabbits with uranium nephritis.

Marriott and Howland (5) found an increase in the inorganic phosphate of the blood in nephritis in man, the increase being sufficient to account for the acidosis. On the other hand, Denis (6) obtained normal values for the inorganic phosphate concentration of the blood in uranium nephritis in rabbits. She did not follow the course of the nephritis for any great length of time and it is possible that the samples were obtained before any marked phosphate retention had begun. We shall show that dogs excrete the normal or more than the normal amount of phosphate during the early stage of uranium nephritis.

Goto (7) observed that the injection of uranium nitrate into dogs causes a fall in the CO_2 -combining power and the pH of the blood, and an increase in the urea.

Cushny (8) says that acid, *i.e.* ionic hydrogen, is the most toxic of the known urinary constituents. The nitrogenous substances excreted in the urine are only mildly toxic. In the present work we have compared the changes in the blood with the amount of excretion of phosphates, organic acids, basic substances, and sugar by the kidney in uranium nephritis in order to determine the changes in the urine which might account for the development of the decrease in CO_2 -combining power and pH, and the increase of the sugar in the blood. Some variations in the acidosis have led us to consider the possible loss of acid through the alimentary tract, particularly from the stomach.

EXPERIMENTAL.

Dogs, mostly female, were fasted for at least 24 hours before the blood and urine were collected for control analyses. The fasting was continued throughout the course of the experiment. The dogs were kept in large metabolism cages, and the urine was collected in bottles containing toluene. The cages were kept scrupulously clean and every precaution was taken to prevent contamination and putrefaction of the urine. Whenever possible, the urine analyses were checked by analyzing, separately, urines obtained by catheterization. Arterial blood was drawn from the heart for the blood analyses.

The acid of the urine was titrated, after the addition of an excess of solid neutral potassium oxalate, with 0.1 N NaOH, using phenolphthalein as the indicator. The phosphates were determined by titration with standard uranium acetate solution. The organic acids of the urine were determined by the method of Van Slyke and Palmer (9), the pH of the urine by Clark's indicator method (10), and the urinary sugar by the method of Shaffer and Hartmann (11). The ammonia of the urine was estimated by the aeration method of Folin (12). In the case of the blood analyses, the CO_2 -combining power was determined by the Van Slyke (13) method, pH by Cullen's (14) method, and non-protein nitrogen and sugar by the methods of Folin and Wu (15).

The excretion of albumin and the increase of the non-protein nitrogen of the blood have served as indications of the nephritic condition. The excretion of albumin began very soon after the

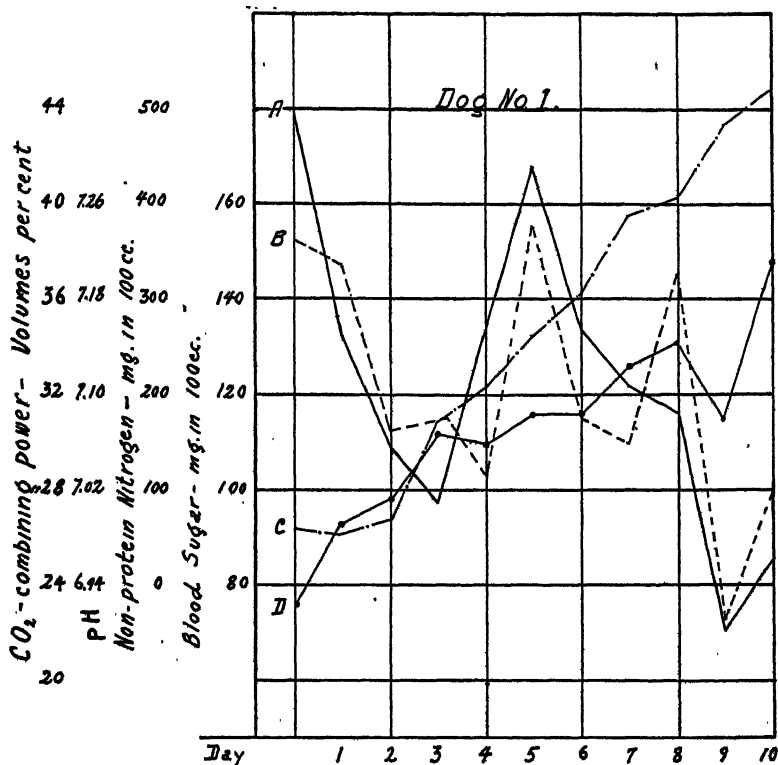


CHART 1. Dog 1, female, weighing 12.8 kilos, received an injection of 76.8 mg. of uranium acetate. This chart shows the variations which occurred as a result of this injection in (A) the CO₂-combining power of the blood, (B) the pH of the blood, (C) the non-protein nitrogen of the blood, and (D) the blood sugar.

uranium acetate injection, being present in specimens of urine collected 3 hours following the injection. Albumin disappeared from the urine when the marked oliguria developed later in the course of the nephritis.

One of the most noticeable effects of uranium intoxication in dogs is the very prompt development of acidosis as shown by the fall in the CO_2 -combining power and pH of the blood. The fall in the CO_2 -combining power of the blood in 24 hours was more than 40 per cent in Dog 21, and in every case it was comparatively great. In the case of Dog 18, the fall was only slightly more than 8 per cent. The change in pH was likewise very noticeable within 24 hours after the injection except in Dog 19 in which it remained unchanged. The fall in pH within the first 24 hours was, in one case (Dog 23), greater than 0.3. The development of acidosis and changes in the sugar concentrations of the blood were followed more closely in Dogs 21 and 22. In both these animals there was a definite fall in the CO_2 -combining power and pH of the blood within 3 hours after the injection. The appearance of relatively large amounts of sugar in the urine was likewise observed in both animals soon after the injection. A pronounced increase in the blood sugar was observed, however, in only one of the dogs (Dog 22).

The change in the character of the urine after the administration of uranium is most interesting. Except in Dogs 18 and 22 there was observed an increased excretion of phosphates during the first few days of the intoxication. This increase was considerably more than 100 per cent for the first 24 hours of the nephritis in the case of Dog 23 which eliminated 0.519 gm. of P_2O_5 on the control day and 1.213 gm. on the 1st day after the injection. Dog 22 passed through the stage of high phosphate excretion so rapidly that the analysis of the composite 24 hour urine specimen did not reveal the excessive excretion of phosphate which was observed in the urine specimens collected 6 and 9 hours after the uranium was administered.

The titratable acidity and hydrogen ion concentration of the urine are markedly decreased at the time of high phosphate excretion, these changes being simultaneous with the fall in the CO_2 -combining power and pH of the blood. Since the phosphate and pH of the urine are increased while the titratable acidity is decreased, there is obviously a greater loss of base from the body during this period than in the normal period; that is, more of the phosphate is excreted as the basic phosphate. There was no evidence of hyperpnea after the administration of uranium

acetate which might have caused a loss of base from the body by a compensatory excretion of alkali to balance the loss of CO_2 from the blood. In order to show these variations in our experiments, we have calculated the percentage of acid phosphate

$$\frac{\text{Cc. 0.1 N acid}}{\text{Cc. 0.1 M phosphate}} \times 100$$

in each specimen of urine (see protocols), assuming that all the acid was excreted as the acid phosphate.

In the case of Dog 19, the fall in the percentage of acid phosphate excreted was not observed until the 2nd day when it was relatively slight. There was no decrease in titratable acidity in this animal. Dog 18 did not show a decrease either in acid phosphate or in titratable acidity. However, this animal did not develop severe enough nephritis to result fatally. Except for this experiment and the one performed on Dog 23, all other experiments were terminated by the death of the animals. In most cases, the loss of base was most rapid during the first hours following the injection. Both Dogs 21 and 22 excreted a urine alkaline to phenolphthalein 9 hours after the uranium had been administered. Of course, only negligible amounts of acid phosphate were being excreted at this time.

Still another way in which bases are lost from the dog's body is by the excretion of salts of organic acids. Unfortunately, we did not, at first, recognize the rôle which organic acids play in depleting the body of base; therefore, we have complete data only in the case of four dogs. The increased excretion of organic acids varied from 3.51 times the normal in Dog 25 to 6.28 times in Dog 24. These results can leave no doubt in regard to effect produced by the formation of excessive amounts of organic acids. In Table I are presented the results obtained by calculating the loss of base from the body by the various factors during the first 24 hours of uranium nephritis. A comparison of this loss is made with the observed loss of base from the blood as indicated by the fall in CO_2 -combining power. The blood has been regarded as a 0.05 normal solution of base in combination with carbonic acid, protein, and phosphate. These calculations show that the loss of base from the body is greater than can be

accounted for by the change in the CO_2 -combining power of the blood.

It appears that the acidosis occurring early in uranium nephritis is due to the excessive excretion of base, through the kidney, in combination as dibasic phosphate and as salts of organic acids. Ammonia does not account for any portion of the increase in base excretion. As a matter of fact, the excretion of ammonia is always decreased after the injection of uranium acetate. On

TABLE I.

Loss of Base in First 24 Hours after the Injection of Uranium Acetate.

Dog No.	Change in titrat- able acidity of urine.	Excess phosphate excreted — excess phosphate in con- trol experiment.	Loss of base through phos- phate excretion.	Loss of base as salts of organic acids.	Change in alkali reserve of the blood.	Total alkali re- serve lost.	Total loss of base through urine.	Decrease of base in blood.
	cc. 0.1N	cc. 0.1M	cc. 0.06N	cc. 0.06N	vols. per cent CO_2	per cent	cc. 0.06N	cc. 0.06N
17	-6.0	8.1	28.2		-5.04	9.60		71.7
19*	104.0	167.7	127.6		-7.62	15.65		102.2
20	-23.7	38.3	122.4		-15.86	32.20		191.6
21	-1.0	68.6	139.4†		-21.50	41.88		401.3
22	-45.7	-174.0	-256.8		-12.60	25.17		288.4
23	3.0	97.7	127.6	1,263.8	-11.07	23.50	1,391.4	188.4
24	-9.3	30.5	54.2	1,100.4	-10.81	23.08	1,154.6	134.0
25	-25.3	37.0	124.4	380.0	-9.51	21.14	504.4	112.7
26	-22.9	51.9	125.4	987.6	-7.99	16.98	1,113.0	101.0

*Interval represented in this case is from the 15th to the 39th hour following the injection.

†This represents the variation in base excretion as phosphate from control (acid on control day was in excess of its NaH_2PO_4 equivalent).

this account, our calculated values for the loss in fixed base are slightly lower than is actually the case. We have included the data of certain of the earlier experiments in Table I because they show that a considerable amount of base may be lost in combination with the phosphate alone.

The decrease of urinary ammonia indicates its formation in the kidneys as suggested by Nash and Benedict (16). The acidosis as shown by the fall in CO_2 -combining power and pH of

the blood does not increase the ammonia in the urine when the kidney had been damaged by uranium. It has been demonstrated by Russell (17) that the ammonia content of the blood does not increase in clinical nephritis.

Within several days after the onset of severe uranium nephritis, the urine changes in character, becoming scanty in amount and containing very little phosphate, though for a time, the organic acids continue to appear in relatively high concentration. Although our dogs were fasting, there must have been considerable retention of phosphate at this time. As there was no feces excreted in most of our dogs, it appears unlikely that phosphates may have been eliminated by way of the alimentary tract. It seems obvious, therefore, that the acidosis occurring in the later stages of uranium nephritis is due to the retention of acid phosphates. In this connection, it is to be noted that in the dogs which recovered (Nos. 18 and 23), the excretion of phosphate remained relatively high throughout the course of the experiment.

The character of the organic acid eliminated has not been fully investigated, but we believe that they were, for the most part, very weak acids. The pH of the urine was usually comparatively high although the titratable acidity greatly exceeded the amount which could be accounted for by the phosphates. We have employed the method of Van Slyke (18) for acetone bodies in an attempt to determine the presence of these substances in the urines of our nephritic animals, but only traces were found. It may be mentioned that Badzynski and Karczewski (19) have shown recently that the excretion in 24 hours in man of oxyproteic acids may be equivalent to as much as 271 to 376 cc. of 0.1 N acid. Toward the end of many of our experiments, the pH of the urine decreased to such an extent as to indicate the presence of relatively strong organic acids.

A remarkable condition of a partial or almost complete return to normal of the CO_2 -combining power and pH of the blood sometimes occurs. We have observed this in a number of experiments, but for the purpose of economizing space, the results obtained only in the case of Dog 1 are represented in Chart 1. We have had opportunity to study this peculiarity in some detail upon Dog 25. This animal showed a typical onset of acidosis. The CO_2 -combining capacity on the control day was 44.97

volumes per cent and the pH 7.42. Both declined typically and on the 5th day were 29.11 and 7.17, respectively. The next day, the CO_2 had increased to 36.23 volumes per cent and the pH to 7.23. There was a further increase to 44.78 and 7.33 on the following day. Thereafter there was a decline especially in the pH values. The pH fell to 6.98 on the 9th day, shortly before the dog died. The temporary return to normal seems to have been brought about by the loss of acid by way of the stomach. The fluid in the urine bottle on the morning of the 6th day of the experiment had a pH of 2.8. There was other evidence that the animal had been vomiting. Considerable vomitus was likewise eliminated on the 7th and 8th days of the experiment. It seems certain, therefore, that sufficient acid was excreted through the stomach to relieve the acidosis. The facts that the non-protein nitrogen of the blood continued to rise and the elimination of phosphates was decreased to insignificant values during this period of apparent recovery, indicates that the kidney was becoming less efficient as an organ of excretion. Blood taken from the animal several hours before death was found to contain 532 mg. of non-protein nitrogen in 100 cc.

The relation between the pH and the sugar of the blood does not seem to be a constant one. The blood sugar is always high and the pH low for a few days preceding death. On the last day of the experiment, in the case of Dog 25, we recorded a blood sugar value of 400 mg. in 100 cc., the highest concentration which we have observed. The pH of the blood at this time was 6.98. The hyperglucemia occurring in the last stages of uranium nephritis seems to be more or less comparable with the low pH. However, the low pH cannot be regarded as the only factor affecting the blood sugar concentration, for the increased renal permeability characteristic of early uranium nephritis counteracts any tendency toward hyperglucemia, and may at times even result in a diminution of the blood sugar concentration to sub-normal values. In a number of instances a slight hypoglucemia was observed 24 hours after the uranium injection. We have no data pointing to other factors which might influence the blood sugar concentration. It has been suggested that liver injury might be the cause of hyperglucemia. However, artificially-induced liver injury in previously fasted animals usually causes

hypoglucemia. A study of our data gives us the impression that acidosis is at least partially responsible for the hyperglucemia, but the results are too inconsistent to permit of any definite conclusions in this regard.

SUMMARY.

1. In confirmation of much earlier work, acidosis as shown by the CO_2 -combining power and pH of the blood, hyperglucemia, and glucosuria have been observed after the administration of uranium acetate to dogs.

2. In the early stages of the intoxication the acidosis is associated with an increased excretion by the kidneys of basic phosphates and the salts of organic acids.

3. The severe acidosis of the later stages of uranium nephritis is associated with a marked decrease in phosphate excretion.

4. The decrease in urinary ammonia in uranium nephritis lends support to the theory of Nash and Benedict that it is of renal origin.

5. In a number of dogs, we have observed sufficient loss of acid from the stomach by vomiting to relieve partially the acidosis. This effect, however, was a temporary one.

6. Increased renal permeability is probably responsible for the glucosuria occurring early in uranium nephritis.

7. Although some relationship between the degree of acidosis and the glucosuria and hyperglucemia is indicated, it does not appear that this relationship is a quantitative one.

Protocol I.

Dog 17, male, weight 9.0 kilos. Injected 54 mg. of uranium acetate, Feb. 4, 10 a.m.

Date.	Blood analyses.				Urine analyses.*				
	CO ₂	pH	Non-protein N per 100 cc.	Sugar per 100 cc.	Volume.	0.1 N acid.	pH	Phosphates. P ₂ O ₅	Sugar.
	vols. per cent		mg.	mg.	cc.	cc.		gm.	gm.
1924									
Feb. 4	52.51	7.46	31.5	88	310	60.0	6.5	0.445	0.60
" 5	47.47	7.35	38.0	108	275	54.0	7.2	0.503	1.29
" 6	33.88	7.29	86.5	116	240	40.0	7.2	0.484	3.02
" 7	34.21	7.32	131.3	111	400	32.0	6.9	0.296	1.45
" 8	36.98	7.20	179.0	108	Urine lost.				
" 9	38.05	7.22	250.0	143	235	50.8	5.6	0.087	Nil.
" 10					200	46.4	5.6	0.059	"
" 11	33.10	7.24	400.0	170	175	12.0	5.8	0.050	"

*The results of the urine analyses in this and the following protocols are for 24 hour peric
†a cc. 0.1 N titratable acidity.

p cc. 0.1 M phosphate

Protocol II.

Dog 18, female, weight 11.14 kilos. Injected 66 mg. of uranium acetate, Feb. 8, 10 a.m.

Date.	Blood analyses.					Urine analyses.			
	CO ₂	pH	Non-protein N per 100 cc.	Sugar per 100 cc.	Volume. cc.	0.1 N acid. cc.	pH	Phosphates. P ₂ O ₅ gm.	Sugar. gm.
1924	vol. per cent		mg.	mg.					
Feb. 8	48.81	7.47	28.0	97	285	171.0	5.9	1.055	0.59
" 9	44.72	7.32	31.3	105	415	196.0	5.9	0.995	3.12
" 10					620	292.8	6.3	1.527	11.22
" 11	37.10	7.39	52.5	102	810	266.0	6.1	1.200	9.33
" 12	38.13	7.21	70.0	102	850	136.0	6.0	0.901	5.95
" 13	33.54	7.24	81.0	93	620	156.0	5.8	0.723	4.91
" 14	35.93	7.26	101.0	102	410	98.0	5.8	0.334	3.74
" 15	31.50	7.22	119.0	103	300	61.2	5.8	0.267	1.71
" 16	32.98	7.21	112.5	96	370	80.0	5.8	0.336	2.22
" 17					700	119.0	6.1	0.622	3.54
" 18	37.38	7.33	101.0	95	358	63.0	6.1	0.370	1.65
" 19	32.13	7.28	99.0	102	690	83.0	6.8	0.562	1.87
" 20	41.39	7.38	84.0	106	500	62.0	6.7	0.463	0.95
" 22	43.61	7.60	195.0	105	1,100	119.0	6.7	0.917	0.86
" 27	37.24	7.33	193.8	103	2,005	56.0	7.5	1.472	4.44
Mar. 1	41.83	7.36	109.0	106	1,385	127.0	6.9	1.383	1.74

Protocol III.

Dog 19, male, weight 7.84 kilos. Injected 65 mg. of uranium acetate, Feb. 19, 5 p.m.

Date.	Blood analyses.				Urine analyses.						Remarks.
	CO ₂	pH	Non-protein N per 100 cc.	Sugar per 100 cc.	Volume.	0.1 N acid.	pH	Phosphates. P ₂ O ₅	Sugar.	$\frac{a}{p} \times 100$	
1984	vols. per cent		mg.	mg.	cc.	cc.		gm.	gm.		
Feb. 19	48.70	7.38	40.0	89	210	26.0	6.9	0.292	0.16	63.2	
" 20	40.02	7.38	35.0	81	230	74.4	6.5	0.695	0.63	75.9	
" 21	41.08	7.32	62.5	100	450	130.0	6.8	1.483	0.91	62.3	
" 22	35.08	7.37	91.0	103	340	64.0	6.8	0.565	3.84	80.4	
" 23	36.73	7.34	125.0	100	385	35.0	6.6	0.289	1.35	85.9	
" 24					260	38.0	6.2	0.241	1.05	112.1	
" 25	36.80	7.22	240.0	125	250	46.0	<5.8	0.144	0.50	226.6	Contamination with fecal material and vomitus.
" 26	36.08	7.24	278.0	114	60	3.2	7.2	0.058	Nil.	39.0	
" 27	34.38	7.25	357.5	109	130	15.0	5.1	0.043	"	245.5	
" 28	34.12	7.27	383.5	146	200	17.6	5.0	0.068	"	189.2	
" 29	34.71	7.21	442.5	160	275	10.0	6.8	0.138	"	51.5	Died.

Protocol V.

Dog 21, female, weight 11.5 kilos. Injected 92 mg. of uranium acetate, Mar. 3, 10 a.m.

Date.	Blood analyses.				Urine analyses.						Remarks.
	CO ₂	pH	Non-protein N per 100 cc.	Sugar per 100 cc.	Volume	0.1 N acid.	pH	Phos-phates. P ₂ O ₅	Sugar.	$\frac{a}{p} \times 100$	
1934	vols. per cent		mg.	mg.	cc.	cc.			gm.	gm.	
Mar. 3	51.86	7.34	28.6	104	460	184.0	6.3	1.130	0.72	116.3	10 a.m. Normal.
	47.12	7.20	25.0	105	40	5.6	7.4	0.926	0.24	43.7	1 p.m.
	46.15	7.20	27.3	96	125	10.0	7.4	0.262	1.15	27.1	4 "
	43.27	7.10	31.8	105	110	0.0	8.0	0.342	1.26	0.0	7.45 "
" 4	29.86	7.17	33.1	109	500	8.4	7.0	0.921	7.14	64.8	
" 5	32.99	7.12	43.5	108	680	159.0	6.4	1.010	10.10	111.7	
" 6	32.78	7.13	85.5	126	510	69.2	5.0	0.271	2.70	181.6	
" 7	32.96	7.18	127.0	126	No urine.						
" 8	30.31	7.15	152.0	126	150	60.0	4.4	0.061	0.00	697.6	Contaminated with vomitus.
" 10	29.66	7.23	262.5	125	115	22.0	4.5	0.034	0.00	479.2	
" 11	31.26	7.16	320.0	172	170	32.0	5.2	0.057	0.00	675.0	
" 12	30.45	7.21	450.0	240	205	54.0	4.4	0.033	0.00	466.2	
	18.69	7.01	420.0	202	Animal died in coma shortly after this blood was drawn at 3 p.m.						3 p.m.

Protocol VI.

Dog 22, female, weight 13.75 kilos. Injected 110 mg. of uranium acetate, Mar. 6, 8.45 a.m.

Data.	Blood analyses.				Urine analyses.							Remarks.
	CO ₂	pH	Non-protein N per 100 cc.	Sugar per 100 cc.	Volume.	0.1 N acid.	pH	Phos- phates. P ₂ O ₅	NH ₄ N	Sugar.	$\frac{a}{p} \times 100$	
1934	vol. per cent		mg.	mg.	cc.	cc.		gm.	gm.	gm.		
Mar. 6	50.06	7.41	27.8	96	325	195.0	6.5	2.566	0.3700	0.58	53.95	8.45 a.m. Normal.
	44.95	7.21	28.5	112	50	1.3	7.5	0.068	0.0068	0.58	13.58	11.45 "
	40.05	7.17	27.0	130	110	1.0	8.3	0.171	0.0056	1.18	4.15	2.45 p.m.
	37.54	7.18	33.3	126	105	0.0	8.5	0.253	0.0088	1.67	0.00	5.45 "
"	37.46	7.36	47.5	112	280	147.0	5.7	0.838	0.0558	6.26	124.57	9 a.m.
	36.92	7.26	57.0	106	30	14.8	5.3	0.079	0.0096	0.37	133.09	4 p.m.
"	37.04	7.20	111.0	105	10	3.25	5.0	0.015	0.0000		154.03	
"	27.59	7.23	207.0	115	130	45.0	4.9	0.020	0.0740		1,601.30	
"	25.37	7.26	255.0	112	170	60.0	4.6	0.026	0.0600		1,643.70	
"	26.25	7.21	332.5	122	Urine contaminated with vomitus.							
"	29.47	7.11	345.0	146	"	"	"	"	"	"	"	Died.
"	32.12	7.00	385.0	249	"	"	"	"	"	"	"	

Protocol VIII.

Dog 24, female, weight 6.97 kilos. Injected 42 mg. of uranium acetate, Mar. 17, 1 p.m.

Date.	Blood analyses.				Urine analyses.								Remarks.
	CO ₂	pH	Non-protein N per 100 cc.	Sugar per 100 cc.	Vol- ume.	0.1 N acid.	pH	Phos- phates. P ₂ O ₅	NH ₄ N	0.1 N organic acid.	Sugar.	$\frac{a}{p} \times 100$	
1934			mg.	mg.	cc.	cc.		gm.	gm.	cc.	gm.		
r. 17	46.83	7.41	26.2	79	385	97.0	6.7	0.598	0.183	101.6	0.23	115.20	Possibly some vomitus.
18	36.02	7.22	34.0	93	440	87.7	6.0	0.815	0.087	639.0	4.10	76.40	
19	29.25	7.19	69.0	105	290	67.3	6.2	0.388	0.013	261.0	3.00	123.70	
20	29.24	7.05	100.0	115	10	3.2	4.9	0.015				152.38	
21	28.43	7.03	146.1	111	75	15.3	4.4	0.050	0.003	21.6		217.32	Possibly some vomitus.
22	26.21	7.04	199.2	103	35	6.2	5.2	0.030	0.002			147.61	
24	20.24	6.93	262.5	114	125	13.0	4.9	0.080	0.030	40.0		153.84	48 hr. urine.
25	20.89	6.92	370.0	150	70	19.3	4.6	0.065	0.041	16.0		210.93	
26	18.65	6.90	495.0	274	45	4.5	5.5	0.036	0.044			88.75	
									Animal died.				

Protocol IX.

Dog 25, female, weight 6.4 kilos. Injected 38 mg. of uranium acetate, Mar. 25, 12 n.

Date.	Blood analyses.				Urine analyses.								Remarks.
	CO ₂	pH	Non-protein N per 100 cc.	Sugar per 100 cc.	Vol- ume.	0.1 N acid.	pH	Phos- phates. P ₂ O ₅	NH ₄ N	0.1 N organic acid.	Sugar.	$\frac{a}{p} \times 100$	
1934	vols. per cent		mg.	mg.	cc.	cc.		gm.	gm.	cc.	gm.		
Mar. 25	44.97	7.42	27.0	91	55	58.0	6.5	0.448	0.077	75.6	0.00	92.21	
" 26	35.46	7.32	40.0	61	250	32.7	7.2	0.711	0.014	265.6	2.74	32.65	
" 27	34.61	7.23	65.5	113	280	115.4	5.7	0.614	0.063	582.0	3.57	133.41	
" 28	35.12	7.10	111.0	100	180	14.4	6.5	0.156	0.038	315.0	1.25	65.70	
" 29	35.00	7.10	125.0	95								134.21	
" 30	29.11	7.17	191.0	86	25	5.1	4.4	0.027				522.22	Vomit.
" 31	36.23	7.23	250.0	99	100	42.3	2.8	0.058	0.042	39.6	0.00	775.57	"
" 31	44.78	7.33	326.5	111	300	54.6	3.1	0.050	0.185	54.0	0.00		
" 31	38.44	7.23	382.5	131									
Apr. 1	40.62	7.07	425.0	159	280	16.6	4.6	0.070	0.106	22.0	0.00	169.38	Vomit.
" 2	37.44	6.98	532.5	400	150	4.2	6.2	0.046	0.111	10.5	0.00	64.81	Bile present in vomitus.

Animal died about 5 hrs. after the last blood was drawn.

Animal died about 5 hrs. after the last blood was drawn.

Protocol X.

Dog 26, female, weight 7.14 kilos. Injected 42.8 mg. of uranium acetate, Mar. 30, 12 n.

Date.	Blood analyses.				Urine analyses.							Remarks.		
	CO ₂	pH	Non-protein N per 100 cc.		Sugar per 100 cc.	Vol-ume.	0.1 N acid.	pH	Phos-phates. P ₂ O ₅	NH ₄ N	0.1 N organic acid.		Sugar.	$\frac{a}{p} \times 100$
			mg.	mg.										
1934	vol. per cent				mg.	cc.	cc.		gm.	gm.	cc.	gm.		
Mar. 30	47.04	7.36	29.5	103	80	61.4	6.4	0.350	0.0434		86.0	0.24	124.50	
" 31	39.05	7.22	43.0	92	280	38.5	6.8	0.719	0.0102		568.0	3.01	38.00	
Apr. 1	36.84	7.09	66.0	98	425	81.7	6.7	0.687	Trace.		1,256.0	4.49	83.76	
" 2	32.25	7.15	78.5	98	340	70.7	6.0	0.421	0.0080		132.0	2.25	119.20	
" 3	30.25	7.09	116.2	105	225	52.3	5.5	0.239	0.062		140.4	1.29	155.60	
" 4	27.11	7.18	138.8	112	210	38.2	5.6	0.207	0.059		62.0	0.35	130.80	
" 5	25.44	7.10	169.0	103	200			0.223				0.71		
" 6					215	Alkaline.	8.9	0.255			210.0	0.31		
" 7	29.35	7.11	181.2	102	245	"	8.5	0.269	0.085*		237.0	0.24		
" 8	30.68	7.11	200.0	110	320	"	8.1	0.356	0.085*		200.0	0.62		
" 9	30.01	7.29	193.5	121	325	"		0.403	0.090*		122.0			
" 10	33.31	7.21	215.0	125	340	Neutral.		0.353	0.105*		126.0		Fecal contamination.	

*The ammonia determinations were made on bladder urine and calculated for 24 hour excretion.

Note: The animal appeared to be recovering and therefore the experiment was discontinued. Dog died on April 13, after having been placed on a meat diet.

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SYNTHETIC LECITHINS.

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The work on lecithins in this laboratory has brought out the fact that the purest lecithins prepared to date have been not individual substances but mixtures of lecithins differing from one another by the character of the fatty acids entering into the structures of their molecules. The separation of the individual lecithins from the mixture will always remain a difficult problem and there is very little hope that this will be accomplished in the near future. The more promising way to obtain information as to the properties of individual lecithins seems to lie through synthesis. Eventually, this aim will be accomplished, but for the present, many difficulties lie in the way of its achievement. It was therefore concluded to begin the efforts towards synthesis by reconstructing lecithins from lysolecithins. True lysolecithin itself is a mixture of two substances, one containing stearic, and the other palmitic acid.¹ However, the physical properties of these two cannot differ greatly. Much greater physical differences are to be expected in two lecithins, one containing the radical of oleic, and the other that of arachidonic acid.

Four derivatives of lysolecithin have been prepared to date; namely, acetyl, benzoyl, oleyl, and elaidyl lysolecithins. The instructive point obtained from the behavior of these derivatives is that the physical properties, the brown appearance, and the very soft consistency, which one is accustomed to associate with the natural lecithins, are apparently due to the presence in their molecule of the highly unsaturated fatty acid. The physical properties of oleyl lysolecithin lie, as regards consistency, be-

¹ Levene, P. A., Rolf, I. P., and Simms, H. S., *J. Biol. Chem.*, 1924, lviii, 859.

tween those of dihydrolecithin and natural lecithin, and in point of color, resembles dihydrolecithin entirely.

A particular point of interest is the bearing of the reconstructed lecithin on the hemolytic action of lysolecithins. Lysolecithin and lysocephalin were found to possess hemolytic properties.¹ Yet it was difficult to state with absolute certainty that the hemolytic property was due actually to the molecule of lysolecithin and not to a minute quantity of an impurity. In the course of the fractionation carried out in the present work, samples were obtained which contained varying proportions of lysolecithins and it was found that samples which had all the properties of reconstructed lecithin, but contained small traces of the lysolecithin, still possessed hemolytic properties. These properties disappeared only after all lysolecithin was removed. This observation is valuable as it offers a good method, on the one hand, for testing the purity of the reconstructed lecithin and on the other, for corroborating the theory that the hemolytic action is due actually to lysolecithin.

EXPERIMENTAL.

Preparation of Lysolecithins.—The mixture of "stearic" and "palmitic" lysolecithins used in the following syntheses was prepared and purified according to the methods described in two earlier papers on lysolecithins and lysocephalins.^{1,2} It analyzed as follows:

0.0944 gm. substance: 0.1982 gm. CO₂, 0.0868 gm. H₂O, and 0.0141 gm. ash.

0.1748 gm. substance: (Kjeldahl) 3.70 cc. 0.1 N acid.

0.2622 " " : (fusion) 0.0536 gm. Mg₃PO₇.

It contained no amino nitrogen.

Found. (No. 16). C 57.25, H 10.28, N 2.96, P 5.69, ash 14.93.

Calculated. Lysolecithin. $\frac{\text{Amino N}_2}{\text{Total N}_2} = \frac{0}{100}$

Containing stearic acid. C 57.65, H 10.43, N 2.59, P 5.73.

" palmitic " " 56.10, " 10.29, " 2.22, " 6.14.

² Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1923, lv, 743.

Preparation of Acetyl Lysolecithins.—A mixture of "stearic" and "palmitic" lecithins, in which the unsaturated acid grouping is substituted by acetic acid, may be prepared by heating the mixed lysolecithins in a solution of chloroform and pyridine with acetic anhydride, using sodium acetate as catalyst.

1 gm. of lysolecithin, suspended in a mixture of 5 cc. of well dried chloroform and 5 cc. of pyridine (dried by three distillations over barium oxide), was allowed to stand for 2 days at room temperature with 1 cc. of acetic anhydride.³ The mixture was diluted with methylethyl ketone, filtered from the slight precipitate, and the volatile constituents were removed by repeatedly concentrating to dryness under diminished pressure. The residue was dissolved in methylethyl ketone, and on standing, a material separated which in general appearance resembled lecithin. On drying, it formed a white wax-like mass (0.8 gm.), easily soluble in alcohol, warm methylethyl ketone, and warm acetone.

Like all the lecithin derivatives prepared thus far, it forms a well defined white cadmium chloride salt. The latter is insoluble in alcohol, acetone, and toluene, but very soluble in water and chloroform. Suspended in ether, it forms an emulsion, but on the addition of a few drops of water, the salt passes into the aqueous layer. Its subsequent behavior indicates, however, that its solution in water is accompanied by a greater or lesser degree of decomposition. The cadmium salt may be purified by repeated solution in warm 75 per cent alcohol, from which it separates in white amorphous flakes on cooling.

The free lecithin may be recovered by the usual method of treating a chloroform solution of the cadmium salt with methyl alcoholic ammonia.

Material purified in this manner analyzed as follows:

0.1072 gm. substance: 0.2238 gm. CO_2 , 0.0958 gm. H_2O , and 0.0160 gm. ash.

0.0974 gm. substance: (Kjeldahl) 1.80 cc. 0.1 N acid.

0.1949 " " : (fusion) 0.0396 gm. $\text{Mg}_2\text{P}_2\text{O}_7$.

³ The acetic anhydride used was purified by the method of Walton and Withrow (Walton, J. H., and Withrow, L. L., *J. Am. Chem. Soc.*, 1923, xlv, 2690).

0.200 gm. of substance was hydrolyzed with $\text{Ba}(\text{OH})_2$ for acetyl determination. 4.7 cc. of 0.1 N KOH were required to neutralize the distillate.

Found. No. 55.

C 56.93, H 10.00, N 2.58, P 5.66, CH_3CO 10.15.

Calculated. "Acetic stearic" lecithin.

C 57.70, H 10.01, N 2.41, P 5.32, CH_3CO 7.4.

Calculated. "Acetic palmitic" lecithin.

C 56.10, H 9.80, N 2.55, P 5.60, CH_3CO 7.8.

Although one sample of material on analysis showed an amount of acetic acid equivalent to 0.95 mol, four others were consistently high, averaging 1.3 mols.

Benzoyl Lysolecithin.—A lecithin in which benzoic acid was substituted for the unsaturated acid was prepared by melting the mixed lysolecithins and benzoic anhydride in the presence of sodium acetate, for $1\frac{1}{2}$ hours at 80° . The mixture was then cooled, and 15 volumes of ether were added. The precipitate of sodium acetate and unchanged lysolecithin, which separated on standing, was removed by filtration, the ether evaporated, and the residue dissolved in alcohol. From this solution the lecithin derivative was precipitated as the cadmium chloride salt. Using 7 gm. of lysolecithin, 35 cc. of benzoic anhydride, and 10 gm. of sodium acetate, 8.75 gm. of the crude cadmium chloride salt were obtained.

The cadmium salt of this benzoyl derivative is insoluble in absolute alcohol, but may be purified by repeated reprecipitation from its solution in dilute alcohol. Its solubility increases with the water content. It is insoluble in toluene, benzene, chloroform, and ether. Unlike the analogous salt of "natural" lecithins, it does not form a soluble complex with an ether-water mixture, but on the addition of water to an ethereal suspension, the salt passes into the aqueous layer.

The cadmium salt, decomposed in the usual manner with ammonia, yields the free lecithin derivative. The latter is soluble in warm acetone, alcohol, and ether. From very cold acetone it separates as a colorless mass of buttery consistency. This dries to a white wax-like material, which clears gradually on heating, having no definite melting point, however. Two such substances analyzed as follows:

0.1044 gm. substance: 0.2344 gm. CO_2 , 0.0910 gm. H_2O ; and 0.0128 gm. ash.

0.1145 gm. substance: 0.2522 gm. CO_2 , 0.0940 gm. H_2O , and 0.0138 gm. ash.

0.1933 gm. substance: (Kjeldahl) 3.35 cc. 0.1 N acid.

0.1933 " " : " 3.35 " 0.1 " "

0.2900 gm. substance: (fusion) 0.0520 gm. $\text{Mg}_2\text{P}_2\text{O}_7$.

0.2899 " " : " 0.0513 " "

0.1900 mg. substance after hydrolysis similar to that for acetyl determination required for neutralization of the distillate 2.8 cc. of 0.1 N NaOH.

Found. No. 111.

C 61.22, H 9.75, N 2.42, P 5.00.

Found. No. 121.

C 60.06, H 9.18, N 2.42, P 4.93, $\text{C}_6\text{H}_5\text{CO}$ 15.4.

Calculated. "Benzoic stearic" lecithin.

C 61.42, H 9.37, N 2.17, P 4.82, $\text{C}_6\text{H}_5\text{CO}$ 16.26.

Calculated. "Benzoic palmitic" lecithin.

C 60.09, H 9.14, N 2.27, P 5.03, $\text{C}_6\text{H}_5\text{CO}$ 17.11.

Oleyl Lysolecithin.—An unsaturated lecithin containing oleic acid was prepared in the same manner as was the benzoyl substituted derivatives. Oleic anhydride was obtained according to the directions of Holde and Rietz⁴ and melted at 22°C. A mixture containing 10 gm. of oleic anhydride together with 5 gm. of lysolecithin and 1 gm. of sodium acetate was maintained at 90–100° for 1 hour. All ether-insoluble material was then removed and the lecithin precipitated as the cadmium chloride salt (10.2 gm. of crude product). The cadmium salt, after repeated reprecipitations from dilute alcohol, was decomposed with NH_3 and the lecithin isolated in the usual manner by emulsifying the residue with water and precipitating it with cold acetone. Lecithin obtained in this manner is perfectly soluble in warm dry acetone or methylethyl ketone. It dissolves with difficulty in dry ether.

Analysis at this stage indicated that it was still contaminated by lysolecithin and it was therefore repeatedly fractionated from

⁴ Holde, D., and Rietz, K., *Ber. chem. Ges.*, 1924, lvii, 99.

dry ether containing increasing quantities of acetone and finally by fractional precipitation from methylethyl ketone. The more soluble fraction was a perfectly white hygroscopic substance of the consistency of a soft wax, appearing almost crystalline. On heating, it cleared gradually between 50 and 100°, at no time really melting, and eventually decomposed with gas evolution at about 200°.

This substance analyzed as follows:

No. 169. 0.1006 gm. substance: 0.2371 gm. CO₂, 0.0956 gm. H₂O, and 0.100 gm. ash.

0.0980 gm. substance: (Kjeldahl) 1.30 cc. 0.1 N acid.

0.1471 " " : (fusion) 0.0218 gm. Mg₂P₂O₇.

0.2027 " " : (Wijs) 0.6836 gm. iodine.

Found. No. 169.

C 64.77, H 10.63, N 1.86, P 4.12, Iodine No. 33.

Calculated. "Oleic palmitic" lecithin.

C 64.81, H 10.81, N 1.80, P 3.99, Iodine No. 32.7.

Calculated. "Oleic stearic" lecithin.

C 65.55, H 11.01, N 1.73, P 3.85, Iodine No. 31.5.

Hemolytic Activity of Oleyl Lysolecithin.—Through the kindness of Dr. Hideyo Noguchi, the hemolytic activity of this material toward horse cells and also that of the higher fraction (No. 167) (which had separated from methylethyl ketone at room temperature) were compared with that of the original lysolecithin. 1 per cent solutions of all substances were used in the following.

Dilutions.	Amount.	Lysolecithin.	Oleyl lysolecithin.	
			No. 167.	No. 169.
	cc.			
1:10	1.7	C. H.	C. H.	C. H.
	1.0	"	"	Very slight H.
	0.5	"	Sl. H.	No H.
	0.2	"	No H.	" "
1:100	1.0	"	" "	" "
	0.5			
	0.2	Sl. H.	" "	" "
1:1,000	1.0	No H.	" "	" "

C. H. = complete hemolysis; Sl. H. = slight hemolysis; and No H. = no hemolysis.

By further fractionation from methylethyl ketone, a sample of oleyl lysolecithin was separated which showed no trace of hemolysis in 1:10 dilution.

Elaidyl-Lysolecithin.—Elaidic acid was prepared by the action of nitrous acid on oleic acid. Its anhydride was obtained by the same method as that used in the preparation of its isomer, oleic anhydride.⁴ 3 gm. of elaidic anhydride were condensed with 1 gm. of lysolecithin by the technique described above; the ether-insoluble material was separated as usual, and the lecithin isolated through its cadmium chloride salt. The free lecithin separated in white amorphous flakes from solutions in alcohol and in ether, and was readily precipitated by dry acetone. On drying, it formed a white powder, which showed no tendency to form the typical waxy masses characteristic of the other lecithin derivatives described. It melted sharply at 220°. Owing to the small amount of material available, it was not possible to purify this further.

This material analyzed as follows:

No. 166. 0.0991 gm. substance: 0.2284 gm. CO₂, 0.0946 gm. H₂O, and 0.0139 gm. ash.

0.1930 gm. substance: (Kjeldahl) 2.45 cc. 0.1 N acid.

0.2895 " " : (fusion) 0.0382 gm. Mg₂P₂O₇.

0.2007 " " : (Wijs) 0.065 gm. iodine.

Found. No. 166.

(Ash-free.) C 66.45, H 11.28, N 1.77, P 3.67, Iodine No. 32.

Calculated. "Elaidic palmitic" lecithin.

C 64.81, H 10.81, N 1.80, P 3.99, Iodine No. 32.7.

Calculated. "Elaidic stearic" lecithin.

C 65.55, H 11.01, N 1.73, P 3.85, Iodine No. 31.5.

ON WALDEN INVERSION.

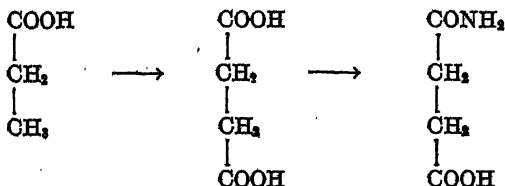
III. OXIDATION OF OPTICALLY ACTIVE THIOSUCCINIC ACID AND THIOSUCCINAMIDE TO THE CORRESPONDING SULFO ACIDS.

BY P. A. LEVENE AND L. A. MIKESKA.

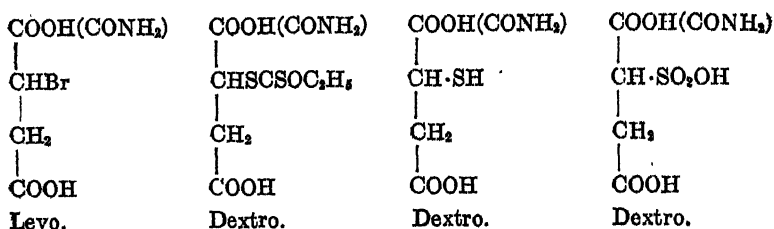
(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 22, 1924.)

The considerations which led to the present investigation were discussed in the first paper of the series. In that paper, the result of the oxidation of optically active secondary mercaptans into the sulfonic acids was reported. This oxidation brought about a change of direction of the optical rotation. The second paper dealt with oxidation of the 2-thiopropionic (thiolactic) acid into the corresponding sulfonic acid. On that occasion, the oxidation remained without influence on the direction of rotation. In the present paper, the results of oxidation of thiosuccinic into sulfosuccinic acid and of thiosuccinamide into sulfosuccinamide are reported. Succinic acid differs from propionic acid by the presence of a second negative group in the molecule. On the other hand, succinamide differs from succinic acid in the polarity of one of the terminal carbon atoms, as is obvious from the following representation.



In both of these substances, the oxidation of the thio acid into the sulfonic acid remained without effect on the optical rotation. The series of reactions was as follows:



There is one point of divergence in the behavior of bromopropionic and bromosuccinic acids which is worthy of note. The bromolactic acid showed only a slight tendency towards racemization, so that the preparation of the optically active thiopropionic and sulfopropionic acids proceeded without difficulty. In the case of the succinic acid, when the directions of Biilmann were followed, the xanthosuccinic and thiosuccinic acids were racemized to such an extent that the sulfo acid prepared from them was totally inactive. In order to obtain the optically active substances, two precautions had to be taken; one during the substitution of the bromo acid by potassium xanthate and the other during the oxidation of the thio acid with bromine. It was found necessary to maintain the first reaction at low temperature, practically at 0°C., and the second reaction had to be carried out at nearly the neutral point. For this purpose, the solution was buffered with an excess of solid barium carbonate.

The composition of the barium salt of the sulfosuccinic acid always showed slight disagreement with the theory and, therefore, the sodium and the acid potassium salts of the sulfonic acid were prepared. The latter gave more satisfactory analytical results.

d-Xanthosuccinic Acid.

The xanthate was prepared according to the directions of Einar Biilmann¹ for the corresponding racemic form. 60 gm. of *l*-bromosuccinic acid ($[\alpha]_D^{20} = -73.62^\circ$) were dissolved in 300 cc. of water and cooled to -5°C . The acid was neutralized with 32.18 gm. of sodium carbonate, whereupon 53.25 gm. of potassium xanthate were added. The mixture was allowed to

¹Biilmann, E., *Ann. Chem.*, 1905, cccix, 369.

stand at 0°C. for 8 days. A few drops of HCl were then added and the solution was filtered. The cooled filtrate was then treated with 122 cc. of cold concentrated hydrochloric acid. On further cooling, the xanthate crystallized out. It was purified by recrystallizing from water. Yield 69 gm. It had an optical rotation of

$$[\alpha]_D^{20} = \frac{+2.16^\circ \times 100}{1 \times 3.46} = +62.43^\circ$$

0.2018 gm. substance: 0.14110 gm. BaSO₄.

C₈H₁₀O₈S₂. Calculated. S 26.92.

Found. " 28.01.

d-Thiosuccinic Acid.

37 gm. of *d*-xanthosuccinic acid ($[\alpha]_D^{20} = +48.00^\circ$) were dissolved in 400 cc. of absolute alcohol, cooled to -5°C., and then treated with 115 cc. of concentrated aqueous ammonia. The mixture was allowed to stand at 0°C. for 7 days. Most of the solvent was then removed under reduced pressure. The residue was rendered alkaline with ammonia, and the xanthoamide was removed by extraction with ether. The greater part of the excess of ammonia was removed from the residue by concentration under reduced pressure. The residue was then treated with 35 cc. of concentrated hydrochloric acid and extracted with ether. The ether extract was dried over sodium sulfate. On concentration and cooling, the thio acid crystallized out. It melted at 138°C. and showed a rotation of

$$[\alpha]_D^{20} = \frac{+2.37^\circ \times 100}{1 \times 4.496} = +52.71^\circ$$

In another experiment, a substance was obtained with an optical rotation of $[\alpha]_D^{20} = +68.43^\circ$. The substance analyzed as follows:

0.2004 gm. substance: 0.3256 gm. BaSO₄.

C₄H₄O₂S. Calculated. S 21.33.

Found. " 22.32.

Barium Sulfosuccinate.

3 gm. of *d*-thiosuccinic acid ($[\alpha]_D^{20} = +68.43^\circ$) were dissolved in 30 cc. of H_2O . To this were added 11.72 gm. of barium carbonate (6 equivalents), and the solution was thoroughly cooled. Bromine was then added until it was no longer consumed. The slight excess of bromine was then removed by adding a small amount of thiosuccinic acid. A slight excess of barium hydroxide was then added whereupon the barium salt precipitated at once. The precipitate was washed with a little hot water. It was divided into 2 parts and treated as follows:

The first part was treated three times with hot water and filtered. The precipitate was dried and rotation determined in 10 per cent hydrochloric acid.

$$[\alpha]_D^{20} = \frac{+0.52^\circ \times 100}{1 \times 6.896} = +7.54^\circ$$

As the salt contains 51.11 per cent of barium, the above weight corresponds to 0.3405 gm. of free acid. Therefore

$$[\alpha]_D^{20} = \frac{+0.52^\circ \times 100}{1 \times 3.405} = +15.27^\circ$$

for free acid.

The substance analyzed as follows:

0.0916 gm. substance: 0.7800 gm. $BaSO_4$ (for Ba).

0.1832 " " : 0.1012 " " (" S).

$C_4H_5O_4S_2Ba_2$. Calculated. Ba 51.11, S 8.00.

Found. " 50.11, " 8.32.

The second part of the salt (1.5 gm.) was suspended in 10 cc. of water and 4 cc. of 10 per cent acetic acid. The solution was thoroughly shaken and filtered. The residue was washed with hot water, dried, and the rotation determined in 10 per cent HCl .

$$[\alpha]_D^{20} = \frac{+0.34^\circ \times 100}{1 \times 2.726} = +12.47^\circ$$

The substance analyzed as follows:

0.0909 gm. substance: 0.0887 gm. $BaSO_4$ (for Ba).

0.1837 " " : 0.1028 " " (" S).

$C_4H_5O_4S_2Ba_2$. Calculated. Ba 51.11, S 8.00.

Found " 50.92 " 7.84

Sodium Sulfosuccinate.

5 gm. of *d*-thiosuccinic acid ($[\alpha]_D^{20} = +45.93^\circ$) were dissolved in 50 cc. of water, cooled to $-5^\circ\text{C}.$, and neutralized with 15.84 gm. of sodium carbonate (9 equivalents). Bromine was then added until it was no longer consumed. The total amount of bromine utilized was 16 gm. (15.96 gm. = 6 equivalents). The last trace of bromine was removed by adding a small amount of thiosuccinic acid to the solution. The solution was then treated with absolute alcohol. An oil precipitated, which crystallized on stirring. This was redissolved in a little water and reprecipitated with absolute alcohol. It was then free from sodium bromide. Dried over H_2SO_4 in vacuum. It had a rotation in 10 per cent HCl of

$$[\alpha]_D^{20} = \frac{+0.80^\circ \times 100}{1 \times 5.04} = +15.87^\circ$$

Calculated on the basis of the free acid

$$[\alpha]_D^{20} = \frac{+0.80^\circ \times 100}{1 \times 3.425} = +23.35^\circ$$

The substance analyzed as follows:

0.1000 gm. substance: 0.0734 gm. Na_2SO_4 .

0.2000 " " : 0.1640 " BaSO_4 .

$\text{C}_4\text{H}_5\text{O}_7\text{Na}_3\text{S} \cdot 1\frac{1}{2} \text{H}_2\text{O}$. Calculated. Na 23.71, S 11.00.

Found. " 23.77, " 11.26.

Potassium Sulfosuccinate.

15 gm. of thiosuccinic acid ($[\alpha]_D^{20} = +45.93^\circ$) were dissolved in 150 cc. of water, cooled to $-5^\circ\text{C}.$, and neutralized with 62.1 gm. of potassium carbonate (9 equivalents). Then bromine was added with cooling until it was no longer consumed. The total amount utilized was 50 gm. (6 equivalents). The solution was then evaporated to dryness under reduced pressure, after the excess of bromine had been removed as in the previous experiments. The residue was taken up in cold glacial acetic acid, thoroughly shaken, and then filtered. This was twice repeated. Potassium bromide remained undissolved. The filtrate was

concentrated under reduced pressure. On concentration, a white crystalline substance separated. This was recrystallized twice from water. It showed a rotation in 10 per cent HCl of

$$[\alpha]_D^{20} = \frac{+0.76^\circ \times 100}{1 \times 5.696} = +13.34^\circ$$

Calculated on the basis of free acid

$$[\alpha]_D^{20} = \frac{+0.76^\circ \times 100}{1 \times 3.862} = +19.67^\circ$$

The substance analyzed as follows:

0.1000 gm. substance: 0.0592 gm. K_2SO_4 (for K).

0.2000 " " : 0.1660 " $BaSO_4$ ("S).

$C_4H_4O_7K_2S \cdot H_2O$. Calculated. K 26.78, S 10.96.

Found. " 26.58, " 11.40.

d-Xanthosuccinmonoamide.

The *l*-bromosuccinmonoamide was prepared according to Walden's directions.² The bromo compound showed a rotation of $[\alpha]_D^{20} = -65^\circ$. 20 gm. of this substance were dissolved in 100 cc. of water, cooled to $-5^\circ C$., and neutralized with 5.41 gm. of sodium carbonate. 16.32 gm. of potassium xanthate were then added. The mixture was then allowed to stand for 6 days at $0^\circ C$. A few drops of hydrochloric acid were then added, filtered, and the filtrate was treated with 20 cc. of concentrated hydrochloric acid. An oil separated, which crystallized on cooling. It was recrystallized from water and dried. In this state of purity, it melted at $138^\circ C$. and showed an optical rotation of

$$[\alpha]_D^{20} = \frac{+3.23^\circ \times 100}{1 \times 4.42} = +73.07^\circ$$

The substance analyzed as follows:

0.2000 gm. substance: (Kjeldahl) 8.15 cc. 0.1 N HCl.

$C_7H_{11}O_4NS_2$. Calculated. N 5.9.

Found. " 5.70.

²Walden, P., *Ber. chem. Ges.*, 1895, xxviii, 2770.

d-Thiosuccinmonoamide.

10 gm. of the above xantho derivative were dissolved in 100 cc. of absolute alcohol, cooled to $-5^{\circ}\text{C}.$, and treated with 28 cc. of concentrated aqueous ammonia. The mixture was allowed to stand at $0^{\circ}\text{C}.$ for 4 days. Most of the solvent was then removed under reduced pressure. The residue was rendered alkaline with ammonia and the thioxanthogenamide was removed by extraction with ether. The residue was treated with 20 cc. of concentrated hydrochloric acid. On stirring and cooling, the thioamide crystallized out. It was recrystallized from water. It melted at $133^{\circ}\text{C}.$ and had an optical rotation in water of

$$[\alpha]_D^{20} = \frac{+2.11^{\circ} \times 100}{1 \times 3.528} = +59.80^{\circ}$$

The substance analyzed as follows:

0.1992 gm. substance: (Kjeldahl) 12.40 cc. 0.1 N HCl.

$\text{C}_4\text{H}_7\text{O}_2\text{NS}$. Calculated. N 9.4.

Found. " 8.71.

d-Barium Sulfosuccinmonoamide.

2 gm. of *d*-thiosuccinamide were dissolved in 30 cc. of water. A little barium hydroxide was added which was followed by an addition of a little bromine. The solution was kept alkaline towards Congo red by following each addition of bromine with an addition of barium hydroxide solution. Bromine was added until it was no longer consumed. The slight excess of bromine was removed by the addition of a little thioamide. The solution was rendered neutral towards litmus and the barium salt was precipitated with alcohol. For purification, it was redissolved in a little water and reprecipitated with alcohol.

In 10 per cent HCl, the substance rotated as follows:

$$[\alpha]_D^{20} = \frac{+0.31^{\circ} \times 100}{1 \times 5.30} = +5.85^{\circ}$$

Calculated on the basis of the free acid

$$[\alpha]_D^{20} = \frac{+0.31^{\circ} \times 100}{1 \times 0.3141} = +9.87^{\circ}$$

The substance analyzed as follows:

0.2000 gm. substance: (Kjeldahl) 5.90 cc. 0.1 N HCl,
C₄H₈O₆NSBa. Calculated. N 4.27.
 Found. " 4.13.

ON NUCLEOSIDASES.

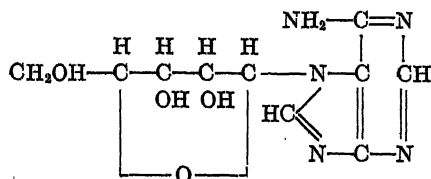
I. GENERAL PROPERTIES.

By P. A. LEVENE, M. YAMAGAWA, AND IONE WEBER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 21, 1924.)

Nucleosidase is one of the enzymes which is concerned with the metabolism of nucleic acid in the organism. Its function is to break up nucleosides of the type



into its component parts. The enzyme was discovered by Levene and Medigreceanu¹ in 1911. At that time, a detailed study of the properties of the enzyme was not attempted nor was it then attempted to isolate the enzyme in a purified condition.

As the work on the structure of nucleic acid progressed, the conviction was reached that certain details of the structure are not accessible to purely chemical analysis and that they may be elucidated through the action of enzymes. Chemical hydrolytic agents are much less discriminating than enzymes. This thought led to the attempt to obtain more detailed knowledge of the conditions of optimal action of the entire group of enzymes concerned in the organism with the breaking down of the molecule of the nucleic acids.

In the course of the work, some intrusions were made into the dynamics of the enzyme action. These intrusions, however, were

¹ Levene, P. A., and Medigreceanu, F., *J. Biol. Chem.*, 1911, ix, 65.

incidental and were made largely with a view of working out a method of standardizing the purity of the material.

The first study of a new enzyme naturally must have the character of a general survey of all conditions affecting its activity, leaving the fundamental analysis of each factor to special investigations. Efforts were first directed towards the analysis of the action of hydrogen ion concentration and of the temperature on the rate of action of the enzyme. After these were established, an attempt was made to analyze the influence of the products of hydrolysis; namely, of ribose and of adenine (adenosin was used as the substrate in all experiments). With this information on hand, the analysis of the influence of variations in the concentration of enzyme and of the variation in the concentration of the substrate on the course of reaction was undertaken.

Fig. 2, expressing the influence of the hydrogen ion concentration, resembles similar curves for other enzymes. The analysis of it will be deferred to another place. It will be mentioned here that it reaches a maximum at pH 7.5.

The optimal temperature was found at 37°C. (Fig. 3). In this connection, it may be mentioned that an irreversible inactivation of the enzyme takes place even at much lower temperatures.

Each component of adenosin, namely ribose and adenine, exerts a retarding influence on the progress of the reaction.

The influence of the concentration of the enzyme and of the substrate was analyzed in greater detail for the reason that the results of these experiments were to serve as guiding points to future work.

The basic assumption made in planning this experiment was that after a certain concentration of the enzyme is reached such that the proportion of enzyme is very large in relation to that of the substrate, then a further increase in the concentration of enzyme will remain without effect on the progress of the reaction.

This expectation was realized. Parallel experiments were made on three different concentrations of substrate. After the concentration of the enzyme reached 1.50 gm. of the crude enzyme per 100 cc. of the solution, a further increase of the enzyme remained without effect. With the enzyme on hand, the limit was reached very rapidly after a sixfold increase in its original concentration. The reason may lie in the fact that the enzyme contained a large

proportion of inhibiting substances. This was to be expected since the enzyme contained all that is contained in the organ plasma.

A second assumption was made to the effect that in the presence of a large excess of enzyme, when its concentration may be regarded constant during the entire course of the reaction, the velocity of reaction should be independent of the concentration of the substrate. Indeed, Table I shows that this expectation

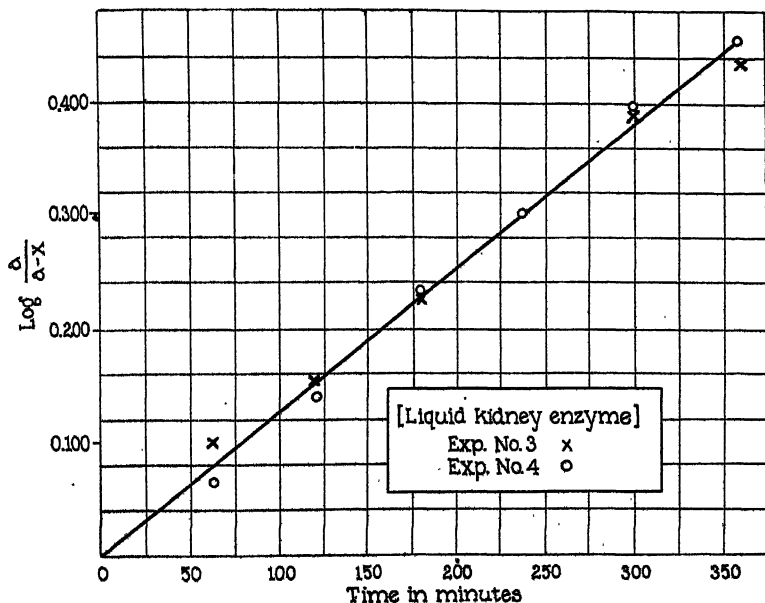


FIG. 1.

was realized. The velocity of reaction remained constant in concentrations of the substrate from 0.050 to 0.500 and 1.5 gm. of enzyme per 100 cc. of solution.

From this observation another conclusion naturally followed; namely, when the concentration of the substrate did not exceed 0.5 gm. per 100 cc. and when the enzyme was present in a sufficient excess, then the velocity of the reaction should follow the mass law in its monomolecular form. As seen from Table II and Fig. 1, this expectation was also realized.

At this phase of the work the question naturally presented itself as to the cause of the course of the reaction at those intervals when it deviates from the monomolecular law. In fact, the latter is of more frequent occurrence than a sustained monomolecular course. Two fundamental assumptions had been considered in order to explain the phenomenon. The first and earliest was that the active molecule undergoing decomposition was the complex substrate-enzyme^{2,3} and hence the course of the reaction was a function of the concentration of that complex. The other postulated that the reaction was dependent upon the concentration of the free catalyst and that the drop in the velocity of the reaction was due to a drop in the concentration of the free catalyst owing to a complex formation between enzyme and the products of hydrolysis.⁴⁻⁹

It seemed to us that the two alternative assumptions might be tested in the following way.

If the second assumption is correct, then it is justifiable to assume that the ratios of the K obtained at different intervals (when the curve follows the mass law for the early intervals) are proportional to the concentrations of the free enzyme. If that assumption is correct, then the dissociation constant of the complex enzyme-inhibitor, calculated from the data of each interval, should have a constant value. The relationship between free enzyme and the complex enzyme-inhibitor is expressed in the following way:

$$\frac{(\text{free enzyme}) (\text{free inhibitor})}{(\text{enzyme-inhibitor})} = \text{constant}$$

Taking E_f = free enzyme; E_t = total enzyme; $E_t - E_f$ = combined enzyme = combined inhibitor; I = total inhibitor; and

² Henri, V., *Z. physik. Chem.*, 1902, xxxix, 194.

³ Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 141.

⁴ Arrhenius, S., *Immunochemistry*, New York, 1907. (See also Euler, H., *Chemie der Enzyme*, München and Wiesbaden, 1920.

⁵ von Euler, H., and Svanberg, O., *Fermentforsch.*, 1919-20, iii, 330.

⁶ Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 471.

⁷ Northrop, J. H., *J. Gen. Physiol.*, 1921-22, iv, 245.

⁸ von Euler, H., and Svanberg, O., *Fermentforsch.*, 1920-21, iv, 29.

⁹ Northrop, J. H., *J. Gen. Physiol.*, 1921-22, iv, 487.

$I - (E_i - E_f)$ = free inhibitor, then the following expression is obtained

$$\frac{E_f(I - E_i + E_f)}{E_i - E_f} = K \quad (1)$$

If the curve of the rate of hydrolysis with relation to the change of concentration of the substrate or with relation to time (original concentration being constant) is a function of the concentration of the complex enzyme-inhibitor, then equation (1) must be fulfilled. Taking the results of Table I and the concentrations of the free enzyme to be proportional to the values of K_1 , then the value of E_f is given for each concentration by the value of the ratio of the initial K to the value of K for that period. The value for E_f during the interval when K remains constant is taken as 100. I is taken to be a multiple of ribose formed during the hydrolysis. Since it was found that inhibitory action of ribose was twice as powerful as that of the base, the value of ribose was multiplied by the factor 1.5. Thus $I = \text{ribose} \times 1.5$; $E_i = 100$; and E_f is taken from Column 5 of Table I. In this manner all data are on hand for the evaluation of the dissociation constant of the complex enzyme-inhibitor. The value was found constant as shown in Table I. If any other value than the one given in Column 5 is attributed to E_f , then the value of K no longer remains constant.

The second alternative view correlates the rate of hydrolysis with the concentration of the complex enzyme-substrate according to the expression

$$\frac{(\text{free substrate})(\text{free enzyme})}{(\text{substrate-enzyme})} = \text{constant}$$

It is seen at a glance that if on this assumption, the values of Column 5 express the proportionality of the concentration of the complex substrate-enzyme, then the values of Column 6 will not be constant but progressively increasing.

Finally, there is a possibility of the substrate acting as an inhibitor. In that case again, the rate of reaction should be proportional to the concentration of the free enzyme and then the following expression should hold:

$$\frac{\text{free enzyme} \times \text{free substrate}}{\text{substrate-enzyme}} = \text{constant}$$

Let E_t = total enzyme, E_f = free enzyme. Hence, $E_t - E_f$ = combined enzyme = combined substrate. Let S_t = total substrate and S_f = free substrate = $S_t - (E_t - E_f)$. Hence,

$$\frac{E_f (S_t - E_t + E_f)}{E_t - E_f} = \text{constant} \quad (2)$$

If E_t is taken as 100, E_f as given in Column 5, S_t from Column 1, calling the first value 100, the second 200, etc., then by equation (2) the values of Column 7 will be obtained. The values of Column 7 seem less satisfactory than those of Column 6.

Thus it seems from the results of Table I, which represents one of five practically identical results, that the more acceptable theory is that the rate of action of an enzyme is proportional to the concentration of the free enzyme. Hence, when conditions are chosen such that the concentration of the enzyme is large with respect to the concentration of the substrate, then the rate of reaction with respect to time should follow the mass law in its monomolecular form. This, as already mentioned, was actually realized in the experiment recorded in Table II.

In Table III an experiment is recorded in which the proportions of enzyme to substrate were such that during the initial intervals the reaction seemed to follow the mass law and later dropped. Assuming that in these experiments also the deviation from the normal course was due to the change in concentration of the free enzyme, it should be possible to determine the constant of dissociation of the complex enzyme-inhibitor. The value, however, is not the same as obtained from experiments recorded in Table I. The discrepancy may be due to the fact that the concentrations of the inhibitor forming in the enzymes were different in the two sets of experiments, some of the inhibitors having their origin in other substances than the nucleoside.

It was therefore realized that a more detailed study of the dynamics of the action of the enzyme nucleosidase has to be deferred until a time when the enzyme will be obtained in a state of greater purity. Thus, both for the practical application and for the theory of the action of the enzyme, it was necessary to direct the efforts towards the purification of the enzyme.

TABLE I.

Showing the Influence of Concentration of the Substrate on the Progress of Hydrolysis.

The quantity of enzyme was constant. $t = 2$ hours.

This table is only one record of several practically identical experiments.

1	2	3	4	5	6	7
Adenosin in original solution.	Sugar in original solution.	Sugar liberated.	$\frac{1}{t} \log \left(\frac{a}{a-x} \right) = 0.4343 K Q$ (10°)	$Q \times 100$	K_1	K_2
mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.				
50	27	5	7.2	100		
100	54	10	7.2	100		
250	135	25	7.3	100		
500	270	48	7.0	100		
1,000	540	74	5.4	75	258	5,925
1,500	810	102	4.9	68	257	6,307
2,000	1,080	128	4.6	63.8	274	6,985
4,000	2,160	180	3.1	43.0	253	5,992

TABLE II.

Experiment 4.

This table represents one of several similar experiments. Liquid extract was used. The concentration of adenosin was 0.5 per cent.

Time (t).	Sugar liberated (x).		$\log \left(\frac{a}{a-x} \right)$	$0.4343 K \times (10^\circ)$
min.	mg.	per cent		
60	0.26	13.5	0.0643	11
120	0.53	28	0.140	11
180	0.80	42	0.235	13
240	0.96	50	0.303	13
300	1.13	59	0.399	13
360	1.24	65	0.455	13

$a = 1.91$.

TABLE III.

Experiments 1 and 2.

Concentration of adenosin = 0.5 per cent.

Powdered kidney enzyme was used in this experiment.

Time (t).	Sugar liberated (x).	$\log \left(\frac{a}{a-x} \right)$	$0.4343 K_1 \times (10^3)$	K_1	K_2
min.	mg.				
30	0.22	0.015	5.0	100	
60	0.42	0.030	4.9	100	
120	0.52	0.037	3.1	62	65
150	0.61	0.044	2.9	58	68
180	0.64	0.046	2.6	52	52
210	0.71	0.051	2.4	48	50

 $a = 6.373.$

EXPERIMENTAL.

Preparation of Enzyme.—In the earlier part of the work the enzyme was prepared in solid form. It was readily soluble in the buffer solution. This form of enzyme seemed advantageous since it permitted the preparation of sufficient material for a large number of experiments. The details of the preparation of the enzyme were as follows:

Preparation of Solid Nucleosidase from Spleen.—The minced organs were extracted with 10 per cent NaCl solution, and the juice was expressed with hydraulic pressure. The juice was concentrated in vacuum to a thick syrup, when it was transferred to a vacuum desiccator and maintained under diminished pressure until the material was dry. It was scraped from the sides of the desiccator and ground in a mortar to a fine powder. This method of drying facilitated the recovery of the dried material, which was brittle and adhered tenaciously to the walls of the desiccator.

Preparation of Solid Nucleosidase from Kidney.—The nucleosidase used in Experiments 1 and 2 was prepared as follows: The minced organs were extracted with Sörenson's phosphate buffer solution (pH 7.0) for an hour at room temperature. The juice was expressed with hydraulic pressure and concentrated in vacuum to a volume of 200 cc. This juice was poured slowly into 10 liters of dry acetone with constant stirring. After the precipitate had settled and the acetone syphoned off, another 10 liters of dry ace-

tone were added and stirred thoroughly. After the precipitate had settled, the acetone was again syphoned off and the precipitate filtered on a Büchner funnel and dried in a vacuum desiccator. The activity and other properties of this material did not differ from those prepared by the first process.

It was later discovered that the enzyme prepared in this manner did not retain its original activity for a great length of time. As a rule, even the fresh material dissolved to the original volume did not possess the original activity. Hence, the later experiments were carried out by the liquid extract prepared in the following way.

The liquid extract was prepared by extracting 2.5 kilos of hashed kidneys with 1 liter of Sörenson's phosphate buffer solution (pH 7.0) for 1 hour at room temperature. The juice was expressed with hydraulic pressure and used immediately.

General Procedure.—The progress of hydrolysis of adenosin was measured by the amount of ribose liberated at given intervals. However, the second reaction product, adenine, interferes with the sugar estimation by the reduction method. The polarimetric method could not be used successfully because the solution of the enzyme was too opaque to permit an accurate reading. Hence it was necessary to devise a method for the removal of adenine from the solution prior to the sugar estimation. This was accomplished by means of phosphotungstic acid. In experiments where the adenosin concentration was not very low, the method gave satisfactory results. Later, however, when the experiments were performed with very dilute solutions, the method failed. The precipitation of the adenine with a saturated solution of mercuric acetate was then resorted to.

General Procedure in the Experiments with the Solid Enzyme.—0.5 gm. of the dry enzyme from spleen and 0.15 gm. of adenosin were dissolved in 25 cc. of Sörenson's phosphate buffer solution. Toluene was used as a preservative. After the solution had been maintained at the indicated temperature for the desired period of time, it was washed into a 150 cc. volumetric flask, phosphotungstic acid was added to precipitate the adenine, and the mixture made up to standard volume with distilled water. After allowing the precipitate to settle overnight in the refrigerator, it was filtered off and the sugar in 60 cc. portions of the filtrate was determined by reduction with Fehling's solution. The figures

given in the table were calculated on the basis of 1 gm. of adenosin. In all experiments, (save those aiming to test the influence of pH), the pH was adjusted to 7.5 by means of the phosphate buffer solution. The sugar was estimated by the method of Griesbach and Strassner.¹⁰

All experiments save those reported in Tables II and III were performed by this procedure.

Procedure of Experiments Summarized in Table II.—0.75 gm. of adenosin was dissolved in 5 cc. of Sörenson's phosphate buffer solution (2/15 molar, pH 7.5), and added to 145 cc. of the liquid kidney extract. The adenosin was omitted for the control. Both were preserved with toluene, and digested for the indicated time intervals at 37°C., 15 cc. samples were pipetted into 200 cc. volumetric flasks, the adenine was precipitated with mercuric acetate, and the solution made up to standard volume. The sugar present was determined by the micro method of Shaffer and Hartmann.¹¹

Procedure of Experiments Summarized in Table III.—24 gm. of kidney enzyme and 3 gm. of adenosin were dissolved in 600 cc. of Sörenson's phosphate buffer solution (pH 7.5). For the control, the same amounts of the enzyme and phosphate solution were taken, the adenosin being omitted. Both were preserved with a small amount of toluene and digested for the indicated time intervals at 37°C.

25 cc. portions were pipetted into 100 cc. volumetric flasks, 30 cc. of saturated mercuric acetate added, and the solution was made up to standard volume. All were allowed to stand in the refrigerator for 2 hours when the adenine was filtered off and the mercury in the filtrate precipitated with H_2S . After filtering, the solutions were brought to a pH of 7.0 by adding solid Na_2CO_3 . For analysis 10 cc. portions were taken. The sugar was determined by the micro method of Shaffer and Hartmann.¹¹

Influence of pH Values.—The experiments were performed with the dry enzyme. The phosphate buffer solution was adjusted to the indicated pH values and the digestion in all experiments interrupted in 4 hours. The results are summarized in Table IV and Fig. 2.

¹⁰ Griesbach, W., and Strassner, H., *Z. physiol. Chem.*, 1913, lxxxviii, 199.

¹¹ Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920-21, xlv, 378.

It is seen from Table IV and Fig. 2 that the optimum action lies at 7.5. There is a much sharper maximum than the one found for the optimum of action of many other enzymes. In view of the

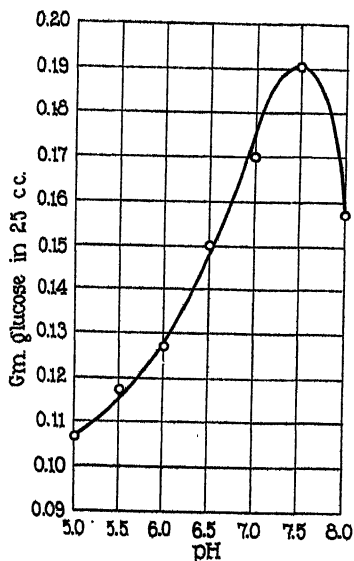


FIG. 2.

TABLE IV.

Action at Different pH Values as Indicated. $T = 42^{\circ}$.

pH	Glucose in 25 cc.	Glucose per gm. adenosin
	mg.	mg.
5.0	32	107
5.5	35	117
6.0	38	127
6.5	45	150
7.0	51	170
7.5	57	190
8.0	47	157

fact that adenosin, which is a glycoside of ribose with a weak base, possesses amphoteric properties, it is not possible to interpret the significance of the curve of Fig. 2 at the present moment. An adequate interpretation will be presented in a later publication.

Influence of Temperature.—The experiments in this direction have only the character of orientation experiments. They were not planned with a view of establishing the temperature coefficient of the action of the enzyme. As will be seen from experiments

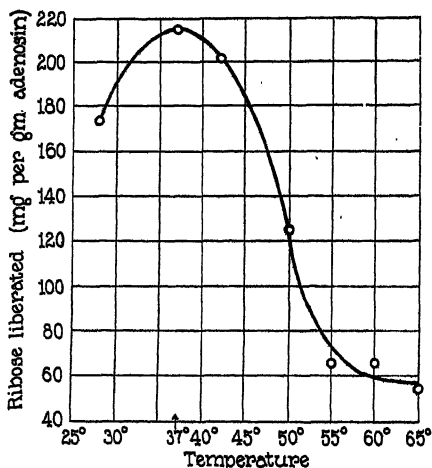


FIG. 3.

TABLE V.

Each sample was digested for 4 hours at the temperatures indicated.

Temperature.	$\text{Na}_2\text{S}_2\text{O}_3$ corrected for control.	Ribose liberated per gm. adenosin (corrected).
°C.	cc.	mg.
28	3.2	174
37	3.9	215
42	3.7	203
50	2.3	125
55	1.2	66
60	1.2	66
65	1.0	55

discussed in a later publication, the enzyme used in these experiments was undergoing spontaneous inactivation even at the temperature of 37°C. Experiments on the temperature coefficient will have to be postponed until a method for the purification of the enzyme is discovered. In Table V and Fig. 3 the milligrams of liberated ribose are plotted against the temperature values.

From the table and curve it is apparent that temperature has its optimum at 37°C. (at the optimal pH 7.5).

Influence of Concentration of Enzyme.—These were planned with a view of obtaining the information required for planning the

TABLE VI.

The usual procedure was followed in these experiments with the exception of the concentration of enzyme.

Enzyme per 100 cc.	Ribose liberated (per gm. of adenosin).		
	0.05 gm. adenosin.	0.10 gm. adenosin.	0.30 gm. adenosin.
mg.	mg.	mg.	mg.
0.25	310	214	100
0.50	465	366	
1.00	516	410	265
1.50	534	427	368
2.00	534	427	380
2.50	534	427	

TABLE VII.

Average of two experiments.

Ribose added.	$\text{Na}_2\text{S}_2\text{O}_3$ corrected for control.	Total ribose.	Ribose liberated from adenosin.	Difference.
mg.	cc.	mg	mg.	mg.
None.	3.8	50	50	
15	4.5	61	46	-4
30	5.5	74	44	-6
60	7.5	101	41	-9

TABLE VIII.

Average of two experiments.

Adenine added.	$\text{Na}_2\text{S}_2\text{O}_3$ corrected for control.	Ribose liberated.	Difference.
mg.	cc.	mg.	mg.
None.	3.80	50	
13.5	3.75	49	-1
27.0	3.60	47	-3
54.0	3.40	45	-5

experiments reported in Tables I, II, and III. From Table VI it seems as if, after a certain concentration of the enzyme has been reached, a further increase in its concentration does not increase

the rate of hydrolysis. However, this result may be due to the great impurity of the enzyme. A more systematic study into the influence of concentration of enzyme will be undertaken after a method is found for its purification.

Influence of the Presence of Ribose.—The only variation from the general method was the addition of the indicated amounts of ribose before digestion. All samples were digested for 6 hours at 37°C.

Influence of Presence of Adenine.—The only variation from the general method was the addition of the indicated amounts of adenine hydrochloride before digestion. All samples were digested for 6 hours at 37°C.

ON NUCLEOSIDASES.

II. PURIFICATION OF THE ENZYME.

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The conclusion reached in the preceding paper was that even with crude nucleosidase, one can select such conditions under which the hydrolysis of the nucleoside will follow the mass law. On the other hand, the crude material seemed to contain a large proportion of inhibitory substances so that it was not possible to demonstrate a direct proportionality between the concentration of the enzyme and the rate of hydrolysis. The results then obtained, however, indicated that with enzyme in a greater degree of purity, one should have less difficulty in adjusting the concentration of enzyme to substrate in such a manner that the rate of reaction would follow the mass law and, further, that the constants of hydrolysis would be directly proportional to the concentrations of enzyme.

These considerations led to work on purification of the enzyme. Unusual difficulties were encountered and the material obtained thus far is still very crude. Yet unmistakable evidence was obtained that the plasma of organs contains substances which act in the sense of inhibitors of the tissue enzymes and by fractional precipitation with colloidal iron, these inhibitory substances can to a certain degree be removed.

Furthermore, it was actually found that with the purer material, it is easier to adjust concentration of the enzyme with respect to the substrate in such a manner that the rate of reaction follows the mass law and that the constants of reaction seem to be proportional to the concentrations of the enzyme.

There is a tendency by many writers to associate catalytic action in living cells with surface action. A point of interest which was brought out in the course of the work is the following.

In the case of our enzyme it was observed that certain adsorbents kept the enzyme so firmly fixed that the latter could not be extracted by water or aqueous solutions of salts at different hydrogen ion concentrations. These solid suspensions of adsorbents containing enzyme were as active as solutions of the same enzyme. Thus it is obvious that enzymes may be so orientated on solid surfaces as to be active. From this possibility, however, it does not follow that they act only on surfaces.

TABLE I.

Time Experiment Using Na_2HPO_4 Extract Concentrated to Minimum Volume.

Used this concentration for No. 1 and one-half concentration for No. 2. 40°C .; 0.5 per cent adenosin.

Time.	No. 1.			No. 2.		
	Change.	Activity.	K	Change.	Activity.	K
hrs.		per cent			per cent	
10	0.08	16	0.00758	0.04	8	0.00362
15	0.11	22	0.00726	0.06	12	0.00370
20	0.14	30	0.00713	0.08	16	0.00327
Average			0.00732			0.00353

TABLE II.

Pancreas S.

10-10* filtrate concentrated to $\frac{1}{2}$ volume for the following experiment. $\frac{2}{3}$ and $\frac{1}{3}$ of this final concentration were used. 30°C .

Time.	Two-thirds concentration.			One-third concentration.		
	Reading change.	Activity.	K	Reading change.	Activity.	K
min.		per cent			per cent	
30	0.12	24	0.0040	0.07	14	0.0022
80	0.28	56	0.0044	0.20	40	0.0027
135	0.38	76	0.0045	0.27	54	0.0025

* 10 per cent dialyzed iron plus 10 per cent alcohol.

It must be added, however, that on employing apparently the same procedure, one obtains a fair degree of purification in one experiment and nearly complete inactivation in another. However, in all experiments, an increase in potency is obtained when certain inhibitory substances are removed by means of dialyzed iron and alcohol.

EXPERIMENTAL.

Extraction of the Enzyme.

The enzyme used in the earlier experiments was prepared by concentrating the plasma of fresh organs under diminished pressure. Frequently, it was found that the dry material prepared in this manner was not completely soluble in water. In such cases, a large proportion of the enzyme remained adherent to the insoluble part and thus was practically lost. In the course of the present work, an extract was used instead of the plasma. 5 pounds of organs were washed free of blood, ground, and extracted with 4 liters of 2 M/15 Sörenson's phosphate solution. The optimum pH for this extraction was found to be 7.0. In the case of the kidneys it was further found that a more active extract was obtained when the organs were allowed to autolyze for 24 hours at 40°C., and, after filtering through cheese-cloth, digesting the juice for 24 hours longer at 40°C. In the case of the pancreas, the extract was inactivated during the second period and consequently only the first autolysis was used.

Purification of the Extract.

For purification of the enzyme, the adsorption method was employed. As adsorbents the following substances were tested: cholesterol, stearic acid, kaolin, aluminum hydroxide ($\text{Al}(\text{OH})_3$), and colloidal iron.

Cholesterol and stearic acid were added in concentrated acetone solution. On dilution of the acetone with aqueous extract, the cholesterol or the fatty acid gathered on the surface of the solution and could be filtered off. All the active material remained in solution. Aluminum hydroxide samples designated by Willstätter and Kraut¹ as A and B were prepared following the directions of these authors, but neither of these samples adsorbed the active principle at pH 7.0. Kaolin was found a very powerful adsorbent but it was difficult to reextract the enzyme from the adsorbent.

Thus, when 1 per cent (by weight) of kaolin was shaken 15 minutes with the enzyme, 12 per cent of the total enzyme content

¹ Willstätter, R., and Kraut, H., *Ber. chem. Ges.*, 1923, lvi, 149.

was left in solution, and 3 per cent of kaolin adsorbed all the active material. It was found that only in the marked acid, or in the marked alkaline regions, could the active principle be re-extracted. Thus, at pH 4.0 and 8.0 the active principle was extracted to the extent of 20 per cent in each case. When the kaolin was extracted with sulfuric acid at pH 4.0 and the sulfuric acid removed with barium hydroxide, the barium sulfate carried down all the active principle.

Most successful was the purification with dialyzed iron and alcohol. By fractional precipitation by means of dialyzed iron and alcohol, it was possible first to remove certain of the inhibiting substances, so that the filtrate had a higher potency than the original solution, and by further addition of iron and alcohol, all the active material was precipitated. The iron and alcohol required to remove the inhibiting substances from individual extracts differed only within narrow limits. As is seen from the tables, the inhibitory substances were removed when 20 per cent (by volume) of iron solution and 20 per cent of alcohol were added to the extract. From the filtrate obtained in this manner a further addition of 40 per cent of iron solution and 10 per cent of 95 per cent alcohol (4.0 cc. of iron and 1 cc. of alcohol to 10 cc. of the filtrate) precipitated all active material.

Extraction of the Enzyme from the Iron Precipitate.

The enzyme adsorbed by dialyzed iron adheres to it very tenaciously. Water and aqueous solutions of glycerol (0.1 per cent), a solution of adenosin (0.5 per cent), and monopotassium phosphate at pH 4.4 failed to reextract the enzyme. The enzyme was extracted successfully by means of disodium phosphate at pH 8.76 or by sodium hydroxide at the same pH. It was sufficient to suspend the iron precipitate in the solution of the disodium phosphate or of sodium hydroxide at pH 8.76 for 30 minutes and to repeat the operation once in order to extract all the enzyme.

Preparation of a Solid Enzyme.

The extracts from the iron precipitate after neutralization can be concentrated to a small volume under reduced pressure, the

TABLE III.

Fractionation of Enzyme Extract (from Kidneys with Sörensen's Phosphate Buffer Solution at pH 7.0) Using Dialyzed Iron and Alcohol.

Determination of maximum with first precipitation on original extract from organs:

Enzyme 1. 3 hours at 40°; 0.5 per cent adenosin; by reduction.

No.	Fe	Alcohol.	Activity.
			<i>per cent</i>
1	10	10	22.1
2	15	10	23.2
3	20	10	33.3
4	25	10	31.1
5	10	20	30.1
6	15	20	31.4
7	20	20	45.3
8	25	20	38.0
9	25	0	28.7

TABLE IV.

Determination of Amount of Iron and Alcohol Necessary to Precipitate All Active Material from Filtrate of Maximum Activity (See above 20-20).*

Enzyme 1. 4 hours at 40°; 0.5 per cent adenosin; by reduction.

No.	Fe	Alcohol.	Activity.
			<i>per cent</i>
1	10	10	14.4
2	20	10	8.6
3	30	10	6.6
4	40	10	0

* 20 per cent dialyzed iron plus 20 per cent alcohol.

TABLE V.

Na₂HPO₄ Extract of Iron-Alcohol Precipitate.

		<i>per cent</i>
1	Before concentration.	20
2	After concentration to $\frac{1}{2}$ volume + diluted 1:2.	44
	Calculated on original.	18

3,500 cc. concentrated to $\frac{1}{2}$ volume in presence of 5 per cent glycerol (and-concentration); diluted 1:2 and run 5 hours at 40°C.; 0.5 per cent adenosin.

		<i>per cent</i>
1	Original.	26
2	Concentrated 1:2; diluted 1:2.	26

temperature of the water bath not exceeding 35°C. It was possible to concentrate the solution of one-fifth of its original value maintaining the full original activity.

From such solutions the enzyme can be precipitated by a large excess of dry acetone. The precipitate was rapidly filtered on suction and dried under diminished pressure over sulfuric acid or calcium chloride. The dry precipitate obtained in this manner contained practically all the active material of the extract and maintained its potency for more than 10 months when the last portion of the material was used up.

Attempts at Further Purification of the Dry Enzyme.

The precipitated enzyme was still a very crude product. It sometimes contained as high as 76 per cent of ash, and the first task was to remove the inorganic impurities. By dialysis this

TABLE VI.

Na₂HPO₄ Extract of the Iron-Alcohol Precipitate Was Concentrated and Precipitated in Acetone.

15 hours at 40°C.; 0.5 per cent adenosin.

		per cent
1	Original extract.	100
2	Concentrated to $\frac{1}{5}$ volume, made to No. 1.	80
3	Precipitated in acetone (made up to No. 1).	88

could not be accomplished since in the course of dialysis, the activity of the original solution was entirely lost. Whether the loss was due to dialysis of the enzyme or to its inactivation is for the present uncertain.

The dialyses in collodion sacs made with a solution containing 60 cc. of ether, 30 cc. of 95 per cent alcohol, 10 cc. of glacial acetic acid, and 8 per cent of Dupont's "parloidin" and clamped with Hofman-frame-one-piece screw clamps were carried out with 5 per cent enzyme against distilled water and against a 4 per cent solution of mannitol. In both cases the contents of the bag lost 60 to 100 per cent of the active material after dialyzing for 24 hours. When less permeable bags were made after Brown the phosphate failed to dialyze.

Fractional precipitation was unsuccessful. When an attempt was made to precipitate the phosphate by lowering the tempera-

ture the enzyme was adsorbed by the crystalline inorganic precipitate. An attempt was then made to precipitate the organic portion of the crude material by bringing the solution to a definite hydrogen ion concentration. On several occasions the procedure was successful and a substance was obtained which contained but 8 per cent of ash and possessed a high degree of activity. It settled out as a flocculent, readily filterable precipitate at pH 1.2.

The precipitate obtained in this manner gave a pink biuret test; no distinct Millon test; and did not reduce Fehling's solution either before or after hydrolysis.

Preparation of Enzyme by Precipitating Phosphate Enzyme Solution at pH 1.2.

20 gm. of Enzyme 19 were dissolved in 200 cc. of water and brought down to pH 1.2 with 1:5 HCl. Centrifuged, washed in acetone, dry ether; dried in vacuum. Yield 1.7252 gm.

Test of Activity.

14 hours at 40°C.; 0.5 per cent adenosin.

Series 3, No. 1; 0.1 gm. of end-concentration, 100 per cent activity.

Activity of Kaolin and Iron-Alcohol Precipitates.

As noted above, difficulty was encountered in extracting the active material from the kaolin precipitate. That the enzyme had not become inactivated was proved by shaking the kaolin precipitate with adenosin for 15 hours at 40°C. The control contained the same amount of kaolin and adenosin. No adenosin was adsorbed by the kaolin alone, but the enzyme adsorbed on the kaolin exerted all its original activity.

In the case of the iron-alcohol precipitate, it was found that the adenosin could be split by the addition of a water suspension of the iron-alcohol precipitate although an aqueous extract was inactive. By way of control, the iron-alcohol precipitate was extracted with adenosin solution, but none was adsorbed.

Analysis of the Solid Enzyme.

It was realized that the material was so crude that the elementary composition of it could give no indication as to the actual composition of the enzyme, yet it was of interest in order to obtain some idea as to the variation in the composition of individual samples. The striking feature of the analytical data is the high nitrogen content of the material which harmonizes with the failure to obtain positive carbohydrate tests on the material. It is also noteworthy that the material containing 76 per cent of ash and the samples with 8 per cent of ash, when calculated on the ash-free basis, possess a nearly constant elementary composition.

TABLE VII.
Analyses.

	P	Calculated on ash-free basis.				Ash.	Activity in 15 hrs.	
		N	S	H	C		mg.	per cent
Series 3, No. 1*..		20.87		6.35	45.05	8.93	200.0	100
PO ₄ , No. 13....	T 16.91							
	I 16.85	11.63	2.66	9.49	40.32	76.71	229.0	22
NaOH, No. 2...	7.72	12.63	3.13	5.37	42.86	50.55	18.4	25
" " 5...	6.61	14.12	2.72	9.35	49.76	60.55	500.0	25

* Material precipitated by adding HCl to pH 1.2 to phosphate enzyme.

Resistance to Acid and Alkalies.

It is characteristic of nearly all the specifically active substances obtained from animal tissues that they are more resistant to acids than to alkalies. It was, therefore, very surprising to find that nucleosidase is more stable in alkalies than in acid. It must be added that the results obtained with acids are not quite consistent. In one experiment, on standing for 2 hours at pH 1.2, the precipitate retained the potency of the original solution; in a second experiment, all the activity was destroyed. On the other hand, a solution standing for 2 hours at pH 10.08 maintained its original potency.

*Resistance of Nucleosidase to Acid and Alkalies.**Experiment 1.*—Precipitation occurred at pH 1.2.

15 hours at 40°C.; 0.5 per cent adenosin.

		<i>per cent</i>
1	10 per cent Enzyme 19.	100
2	10 " " " 19 adjusted to pH 10.08 for 2 hrs.	100
3	10 " " " 19 " " " 1.2 " 2 " (filtrate).	0
4	Precipitate from No. 3.	100

Experiment 2.—Enzyme (23 + 24) 4 gm. adjusted to pH 1.5, let stand 2 hours, made up to 100 cc., adjusted another sample to 2.0. 15 hours at 40°C.; 0.5 per cent adenosin.

		<i>per cent</i>
1	Original enzyme in same concentration.	80
2	Sample at pH 1.5.	0
3	" " " 2.0.	6



ON NUCLEOSIDASES.

III. THE DEGREE OF SPECIFICITY OF NUCLEOSIDASE AND THE DISTRIBUTION OF IT IN VARIOUS ORGANS AND IN VARIOUS SPECIES.

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(Received for publication, May 21, 1924.)

It was stated in the preceding papers that adenosin was the substrate used for testing the action of the enzyme nucleosidase. Adenosin was chosen because of its greater solubility as compared with that of other nucleosides. Even adenosin has so small a solubility that it renders accurate work quite difficult. On the other hand, a theoretical interest is attached to the question of the optimal hydrogen ion concentration for the hydrolysis of different nucleosides. As derivatives of purine bases, nucleosides may possess several dissociation constants depending on the structure of the base. Thus, adenine as an amino-purine should have a fairly strong basic dissociation constant. On the other hand, inosin may have only a weak acid dissociation constant. Hence, if the action of the enzyme is in some way related to the degree of ionization of the nucleoside, then the optimal hydrogen ion concentration for adenosin should be much different from that of inosin. This, however, is not true, and the optimal hydrogen ion concentration is the same for both nucleosides. The analysis of the curves representing the course of reaction of inosin shows rather an abnormal course. There is an initial fall in the rate of hydrolysis which is followed by a rise. The initial fall is, in truth, misleading. It is brought about by the fact that inosin forms a complex with ribose known under the name of carnine, which is more insoluble at a higher hydrogen ion concentration. At a lower hydrogen ion concentration, carnine remains in solution. However, the real optimal condition is practically the same for adenosin and for inosin.

The other question was whether nucleosidase was adapted to act only on purine, ribosides, or on purine glycosides generally. The answer to this question is important from the physiological and chemical view-points, and also from the point of view of usefulness of the enzyme for the work on the chemical structure of nucleic acids. Fortunately, in the course of other work, a hexoside of adenine was prepared. The sugar of this nucleoside is a ketohexose which seems to differ in its properties from the ketohexoses already known. It was found that enzymes quite active with regard to ribosides remained without action on the adenine hexoside.

Of much interest is the action of nucleosidase on nucleic acids. It was hoped to use these enzymes for the preparation of that fragment of the nucleic acid molecule which contains all the components of the molecule save the purine bases. Kossel and Neumann termed it "thymic acid."¹ There was much discussion as to the true chemical nature of the substance. It seemed that by means of nucleosidase, this substance should be readily prepared. The experiment, however, proved that the enzyme which was quite active with respect to nucleosides remained without action when tested on nucleic acid.

Distribution of the Enzyme in the Organs of Animals of Different Species.

In regard to other enzymes of the group of nucleases, it is known that they are distributed differently in the organs of animals of different species and some of them may be entirely absent in certain animals. Already in the older publications of Levene and Medigreceanu² it was stated that nucleosidase was absent in the plasma of the dog's pancreas, and from pancreatic and intestinal juices. The latter finding was corroborated in the present work. On the other hand, it was found that the pancreas of cattle was a very good source of nucleasidase. This finding is of considerable practical importance. The purification of the enzyme is a very difficult process, particularly if it

¹ Kossel, A., and Neumann, A., *Z. physiol. Chem.*, 1896-97, xxii, 74.

² Levene, P. A., and Medigreceanu, F., *J. Biol. Chem.*, 1911, ix, 375.

is prepared from the plasma of an organ with a rich blood supply, such as spleen or muscle. This difficulty is not met with in the case of the pancreas.

EXPERIMENTAL.

TABLE I.

End-concentration: Enzyme 19 = 1 per cent; inosin 0.5 per cent; 15 hours at 40°C.

pH	Activity.
	<i>per cent</i>
6.0	51
6.4	7
6.8	31
7.2	86
7.6	97
8.0	75

TABLE II.

To test the specificity of Enzyme 19. Substrate, adenine hexoside (0.5 per cent). Enzyme 19 = 0.2 gm. per 20 cc. of end-concentration.

Experiment 1.

22 hrs. at 40°C.

No change.

1 dm. tube 0.5 per cent end-reading corrected +0.07.

Experiment 2.

15 hrs. at 40°C.

No change.

1 per cent adenine hexoside +0.14.

Dog Pancreas.

Experiment 1.—To test the nucleosidic activity of pancreas extract. The pancreas excretion itself was diluted 1:2 and filtered. Run 15 hours at 40°C.; 0.5 per cent adenosin.

Pancreas secretion.....No activity.

Experiment 2.—To determine whether or not the intestine contains material which will activate pancreatic juice. Extracted portion of dog duodenum and added to pancreatic juice. Run 48 hours at 40°C.; 0.5 per cent adenosin.

1. Pancreatic juice alone.....No activity.

2. " " + extract from intestine....." "

Experiment 3.—To test dog (terrier) pancreas for nucleosidase. Cut up dog pancreas. After washing free of blood, added phosphate solution at pH 7.5, and digested at 40°C. overnight. 10 cc. of filtrate +10 cc. of 1 per cent adenosin solution were run for 12 hours at 40°C.

Pancreas extract from dog.....No activity.

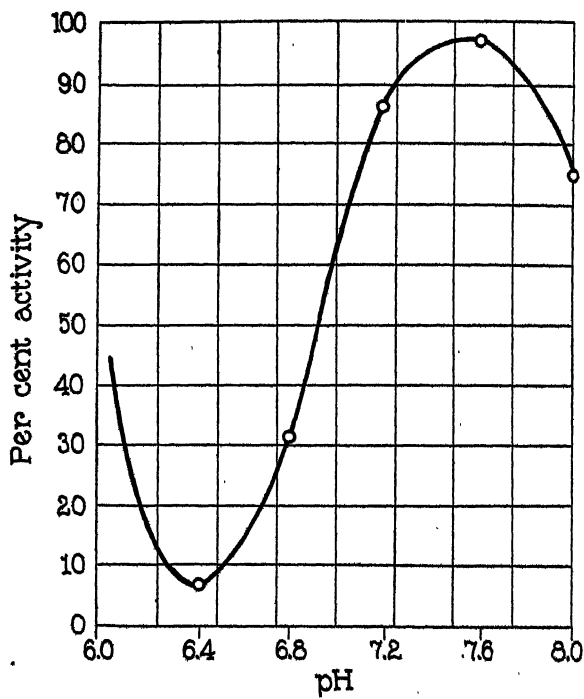


FIG 1.

URIC ACID EXCRETION.*

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(Received for publication, April 17, 1924.)

The excretion of uric acid has been one of the perplexing problems of the physiological chemist for many years and until recently the views and data relating to its elimination have been as vague and varied as those related to the metabolism of this nitrogenous end-product.

It is not the purpose of this paper to add another discussion to the already voluminous literature on this subject, but merely to present some data obtained in an attempt to contribute to the solving of another problem; namely, a method to determine renal insufficiency with a greater accuracy.

The statement that the present renal function tests are inadequate needs no elaboration. Indeed, nothing demonstrates this better than the number of present methods advocated and the continuous search for new ones. Some clinicians use one method, some another, none relying on any one. Many use the data obtained from several tests together with the results of blood analysis and clinical findings to form an opinion concerning renal damage or functional insufficiency, and then a positive statement can only be made after extensive damage has been done. It is usually believed that the reason for this is found

*This work was begun early in 1922 at the University of Wisconsin, but the greater portion of it, especially on the clinical material, was done in the Department of Medicine, University of Minnesota, during the summer of 1922. The author wishes to express his gratitude to Dr. George E. Fahr, whose kindness and help made the work possible. The results herewith published were not presented earlier because of the hope to continue the study with a much larger number and a greater variety of patients, but this not having been possible, they are now presented for the value they may have for other workers.

in the large margin of safety of the normal kidney, thus one kidney can usually be removed without embarrassing the renal activity.

At the time this work was undertaken the opinion was prevalent that uric acid was one of the first substances to be retained in the blood in renal damage. It was thus believed that by overloading the kidney with uric acid, forcing all the renal tissue into activity, and then measuring the excretion of uric acid, a method might be obtained which would not only show slight degrees of renal insufficiency but would show quantitative changes as well. More recently the study of a large series of cases, renal and otherwise, by many different workers, has shown that high uric acid values in the blood may be completely independent of kidney pathology and in nephritis there may be a high retention of other nitrogenous constituents with substantially normal uric acid levels (Folin (1), Feinblatt (2)).

It is altogether reasonable to assume that a substance suitable to measure excretory insufficiency on the part of the kidney should be one that is normally eliminated by that organ. The second requisite that such a substance should fulfill is failure of destruction in the body, in other words, the substance must be quantitatively recoverable after a given amount is administered. If a part of it is destroyed a variable error is introduced into the method which may completely invalidate the results obtained. The low results that the phenolsulfonephthalein method gives at times without demonstrable renal involvement is undoubtedly due to this factor. Uric acid seemed to be a substance, not easily eliminated by the kidney, a normal product of metabolism and eliminated without destruction. The view that uric acid is not destroyed is conveyed by our modern text-books and is based largely upon the findings of Wiechowski (3) who showed in 1909 that when uric acid is injected subcutaneously nearly all of it reappears in the urine and upon the experiments of Schittenhelm (4) who demonstrated that uric acid is not destroyed when it is incubated with extracts of organs at body temperature. The findings of Wiechowski are especially interesting from our standpoint. He injected 1 gm. of uric acid as the sodium salt subcutaneously and recovered 82 to 85 per cent in from 1 to 8 days. More recently, however, results have been obtained

that question these findings. In 1920 Bürger (5) injected uric acid intravenously dissolved in 0.1 N NaOH or in 1 per cent piperazine in amounts of 0.5 gm. He recovered various amounts in the urine in the 24 hours following, ranging from 17.6 to 52.2 per cent. The added recovery on the following days raised these percentages but slightly. During the same year Griesbach (6) failed to recover a considerable percentage of uric acid injected intravenously in six out of seven cases. In one case he recovered more than injected. These authors from their results concluded uricolysis. After the work of this paper was started in 1922, the results of Thannhauser and Weinschenk (7) were published. These authors recovered from 25 to 93 per cent of the uric acid injected intravenously in 1 gm. amounts as the monosodium salt. They found that in the normals there was practically no increased elimination after 24 hours after injection while in individuals with gout the elimination was prolonged over 4 or 5 days. In one of their gout cases only 15 per cent of the injected amount was eliminated during the 1st day which gradually rose to 81.9 per cent on the 4th day. These experimenters also found that the uric acid level in edematous tissue fluids rose quite slowly after intravenous injection, 8 hours elapsing before equilibrium took place.

It was deemed advisable to continue the study of elimination of uric acid under high blood levels using the more accurate methods of uric acid estimation developed during the last few years in this country.

Methods.

The uric acid was determined in the blood, urine, and the solution to be injected according to the method of Benedict and Franke (8), using the arsenophosphotungstic acid color reagent. The regular Folin-Wu method of blood precipitation was modified according to the method of Pucher (9) who showed that the original technique gave only a 75 per cent recovery of added uric acid in blood and that heating before filtering gave a 93 per cent recovery.

The uric acid used was a preparation of Merck's and was precipitated twice with dilute HCl from a solution of the uric

acid in 0.1 N NaOH. After thorough washing by suspension in distilled water the uric acid was dried over H_2SO_4 in a vacuum desiccator.

For intravenous injection the monolithium urate was formed by interaction of uric acid with the proper amount of Li_2CO_3 . This solution was made isotonic with glucose. The following proportions were used.

Uric acid.....	1.00 gm.
Li_2CO_3	0.28 "
Glucose.....	1.35 "
Water up to.....	100.00 cc.

This solution was made up just before use with sterile distilled water, boiled for exactly 2 minutes, the lost water added, and injected when cooled to body temperature. The reaction of the solution was approximately pH 7.4. The uric acid content was determined colorimetrically on a portion and the amount injected based upon this estimation. Approximately 12 mg. of uric acid were injected per kilo of body weight.

The subjects were placed on a controlled amount of food intake during observation based upon their usual diet for that period. An attempt was made to control the water intake so as to be about 2,000 cc., but this was not always successful in the hospital wards. Upon the day of the test the morning meal was omitted and the injection or ingestion of the uric acid was started about 9.00 a.m. after a blood sample was taken. The injections were made by the gravity method in the vein of the forearm at the rate of about 10 cc. per minute. The subject received one glass of water at the end of the injection and one 2 hours after when another blood sample was taken. Urine samples were taken for the 24 hours before, 1 hour after, at the end of 3 hours, and 24 hours after. Toluene was added as a perservative and the urine kept in an ice chest.

RESULTS.

The effect that ingested uric acid has on the blood level has never been clearly demonstrated and it was thought advisable to ascertain whether this would be a method of raising the blood uric acid and so adapt itself to our problem. Only a few pre-

liminary attempts on normals were made, the results of which are shown in Table I. NaHCO_3 was given in a few instances to insure solution of the uric acid in the intestine. The results show that the increments of increase were small if not insignificant. Only in one case was the increase appreciable and that was only with a massive dose during thorough alkalization. The results of the

TABLE I.
Ingestion of Uric Acid.

	Uric acid per 100 cc. blood.			Excretion of uric acid in urine.			
	Before.	2 hrs. after.	Difference.		Volume of urine.	Liter.	Total.
	mg.	mg.	mg.		cc.	mg.	mg.
M. G. B., male, age 22, weight 64.4 kg. 5 gm. uric acid suspended in water.	4.00	4.10	0.10	24 hrs. before.	1,640	241	396
				24 " after.	1,550	255	405
				2nd 24 hrs. after.	1,520	276	420
M. G. B., male. 10 gm. uric acid + 20 gm. NaHCO_3 .	3.90	4.15	0.25	24 hrs. before.	1,590	195	310
				24 " after.	2,000	199	398
				2nd 24 hrs. after.	1,650	252	420
A. E. K., male, age 25, weight 67.3 kg. 10 gm. uric acid + 10 gm. NaHCO_3 .	3.60	3.70	0.10	24 hrs. before.	1,750	206	360
				24 " after.	1,980	207	410
				2nd 24 hrs. after.	1,500	266	400
A. E. K., male. 15 gm. uric acid + 10 gm. NaHCO_3 . 10 gm. NaHCO_3 1 hr. before and 10 gm. 1 hr. after.	3.80	4.70	0.90	24 hrs. before.	1,580	206	325
				24 " after.	2,100	295	620
				2nd 24 hrs. after.	1,650	267	433

urinary output were similar in nature. This method of attempting to increase the blood level of uric acid was therefore not adaptable to our problem and further studies were not made.

Table II shows the changes in normal individuals in the blood level and urinary output after intravenous injection of uric acid. The subjects were medical students normal in every

TABLE II.
Intravenous Injection of Uric Acid in Normals.

Excretion of uric acid in urine.										
	Uric acid per 100 cc. blood.			Difference.	Vol- ume.	Liter.	Total.	Per hr.	Recovery.	
	Before.	2 hrs. after.	mg.						mg.	per cent
R. G. H., male, age 30, weight 71.7 kg. 935 mg. uric acid in- jected.	mg.	mg.	mg.	cc.	ml.					
	4.05	5.65	1.60	1,700	182	310	12.9			
				1,630	197	325	13.5			
				1,810	486	880	36.7			
				1,720	205	345	14.3			
E. C. M., female, age 24, weight 59.0 kg. 770 mg. uric acid injected.				1,600	225	360	15.0	570	61	
	3.87	5.60	1.73	1,310	281	368	15.3			
				1,460	267	388	16.1			
				1,390	661	919	38.3			
				1,280	293	375	15.6			69
M. G. B., male, age 22, weight 64.4 kg. 841 mg. uric acid injected.				1,430	252	360	15.5	531		
	3.64	6.55	2.91	1,500	213	320.0	13.3			
				223	334	74.6	74.6			
				196	410	80.8	40.2			
				1,260	517	652.0	31.0			
A. E. K., male, age 25, weight 67.3 kg. 1,000 mg. uric acid. injected.				1,470	211	310.0	12.9			
				1,679		807.0		487	58	
	4.10	5.66	1.56	1,800	200	360.0	15.0			
				200	343	68.6	68.6			
				210	336	70.5	35.8			
				1,440	471	679.0	32.3			
				1,720	238	411.0	17.1			
				1,850		818.0		458	45.8	

respect. They were up and around doing their usual work except that they rested in a recumbent position for the 2 hours following injection. They differ in this respect from the subjects in the following observations who were all confined to their beds throughout the period. The rise in the blood uric acid level at the end of 2 hours was about 2 mg., although there was no great uniformity. Practically all the uric acid that could be recovered from the urine was eliminated during the first 24 hours following injection, there being no definite increase over normal the second 24 hours. Small changes that did occur after 24 hours fall within the error of day to day variation. The recovery ranged from 45.8 to 69 per cent. None of these subjects had any discomfort or showed any symptoms that might have been attributable to the injected uric acid.

Table III shows the results obtained in a similar observation on a series of patients who did not have demonstrable renal disturbances. The results are similar except that the percentage recovery is somewhat lower and the variation greater. This may merely be due to the fact that a much larger number of subjects were studied.

Table IV shows the results on subjects where definite renal insufficiency could be demonstrated. Although the number of cases is altogether too small upon which to base any definite conclusions, the results show a definite retention of uric acid. This is evident in most cases, not only by a very definite retention as shown by the blood level at the end of 2 hours, but also by the delayed elimination extending over 24 hours. Case 1 is an interesting example of marked retention of uric acid although the initial blood level was normal. No renal insufficiency was shown by the phenolsulfonephthalein test or by the blood chemistry. Case 2 is one that probably showed no renal pathology, but showed an insufficiency by the uric acid test due to cardiac decompensation. Case 3, one of essential hypertension (blood pressure 210/160), showed definite retention, but whether this was due to an arteriosclerotic kidney or to functional changes is difficult to tell. Here there were other positive findings of insufficiency. Cases 3 and 4 were of patients with chronic nephritis, one of these, Case 3 with marked edema, and the other without edema, yet the uric acid elimination was approximately the same in both.

TABLE III.
Intravenous Injection of Uric Acid in Diseases Other than Renal.

	Excretion of uric acid in urine.									
	Uric acid per 100 cc. blood.		Differ- ence.	Vol- ume.	Liter.	Total.	Per hr.	Recovery.		
	Before.	2 hrs. after.						mg.	mg. per cent	
Case 1. D. P., female, age 17, weight 37.2 kg. 486 mg. uric acid injected. Neurasthenia.	3.64	6.36	2.72	620	222	137	5.7			
				120	332	40	40.0			
				480	125	60	30.0			
				860	176	141	6.7			
				1,460		240		104	21.3	
Case 2. V. P., male, age 50, weight 48.9 kg. 639 mg. uric acid injected. Asthma.	3.11	5.00	1.89	1,330	166	211	8.8			
				220	250	55	55.0			
				120	246	29	14.7			
				1,000	286	286	13.6			
				1,340		370		159	25.0	
Case 3. C. B., female, age 28, weight 56.6 kg. 739 mg. uric acid injected. Optic neuritis, brain tumor.	3.66	5.40	1.74	830	310	257	10.0			
				220	256	56	56.0			
				165	352	58	29.0			
				1,210	361	437	20.8			
				1,595		551		294	39.9	
Case 4. J. H., male, age 13, weight 37.2 kg. 1,000 mg. uric acid injected (26.9 mg. per kg.). Asthma.	3.03	6.00	2.69	940	182	171	7.1			
				18	577	14	14.0			
				260	473	130	65.0			
				1,550	320	496	23.6			
				1,828		639		468	46.8	

se 5. S. B., male, age 47, weight 70.5 kg. 914 mg. uric acid injected. Gastric ulcer, out.	5.24	9.08	3.84	24 hrs. before. 1 hr. after. 2 hrs. " 1st. 21 " " 3rd. 24 " " ; total.	650 190 110 530 830	240 272 305 357	156 52 34 189 264	6.5 52.0 17.0 9.0	108	11.9
se 6. M. G., male, age 36, weight 59.9 kg. 782 mg. uric acid injected. Gastric ulcer.	3.14	4.96	1.82	24 hrs. before. 1 hr. after. 2 hrs. " 1st. 21 " " 3rd. 24 " " ; total.	700 180 150 480 810	163 244 143 448	114 44 22 215 381	4.7 44.0 11.0 10.2	267	34.3
se 7. A. J. B., male, age 47, weight 85.3 kg. 1,115 mg. uric acid injected. Syphilis of the central nervous system.	3.62	6.23	2.61	24 hrs. before. 1 hr. after. 2 hrs. " 1st. 21 " " 3rd. 24 " " ; total.	1,850 500 460 1,100 2,060	94 300 213 315	173 150 98 347 595	7.2 150.0 49.0 16.5	422	37.8
se 8. R. B., male, age 29, weight 54.4 kg. 715 mg. uric acid injected. Chronic gas- tritis.	4.00	5.56	1.56	24 hrs. before. 1 hr. after. 2 hrs. " 1st. 21 " " 3rd. 24 " " ; total.	450 150 10 500 660	143 230 150 444	64 35 2 222 258	2.7 35.0 1.0 10.6	194	27.0
se 9. D. H., female, age 15, weight 30.8 kg. 403 mg. uric acid injected. Hyperten- sion. Blood pressure 206/ 143, left ventricular hyper- trophy.	4.16	5.52	1.36	24 hrs. before. 1 hr. after. 2 hrs. " 1st. 21 " " 3rd. 24 " " ; total.	1,020 170 60 1,000 1,230	190 167 205 264	194 28 12 264 304	8.1 28.4 6.1 12.6	111	27.5

TABLE III—*Concluded.*

	Excretion of uric acid in urine.									
	Uric acid per 100 cc. blood.				Vol- ume.	Liter.	Total.	Per hr.	Recovery.	
	Before.	2 hrs. after.	Differ- ences.						mg.	per cent
Case 10. H. R., male, age 35, weight 63.4 kg. 828 mg. uric acid injected. Gastric neurosis.	mg.	mg.	mg.		cc.	mg.	mg.	mg.		
	4.00	6.86	2.86	24 hrs. before.	2,000	73	147	6.1		
				1 hr. after.	230	230	53	53.0		
				2 hrs. " 1st.	200	400	50	25.0		
				21 " " 3rd.	1,770	3	537	25.6		
				24 " " ; total.	2,100		640		439	59.8
Case 11. A. A., male, age 49, weight 93 kg. 1,215 mg. uric acid injected. Gastric ulcer.				24 hrs. before.	880	166	156	6.5		
	4.54	6.35	1.80	1 hr. after.	100	390	39	39.0		
				2 hrs. " 1st.	85	320	27	13.5		
				21 " " 3rd.	970	380	369	17.5		
				24 " " ; total.	1,155		435		279	23.0
Case 12. T. R., male, age 49, weight 63.5 kg. 830 mg. uric acid injected. Multiple neuritis (alcoholic?).				24 hrs. before.	1,840	136	251	10.8		
	4.50	6.00	1.50	1 hr. after.	170	261	44	44.0		
				2 hrs. " 1st.	90	234	21	10.5		
				21 " " 3rd.	2,920	193	564	26.9		
				24 " " ; total.	3,180		630		379	45.7

Table V shows a series of cases where no renal insufficiency could be demonstrated, but where there was a question of possible functional impairment. Of this series Case 3 is interesting as a repetition of Case 1, Table IV, after the acute nephritis had apparently cleared up. Here the blood retention was 2.80 after the nephritis as compared with 6.36 during the acute stage and an elimination recovery of 48.4 per cent after as compared with 21.6 per cent for 24 hours during the attack. Case 4, a preeclamptic toxemia, showed a retention that pointed definitely to at least functional impairment of the kidney.

DISCUSSION.

Other workers who have injected uric acid intravenously and thus obtained higher blood levels have not dwelt upon the question of whether or not symptomatic effects were produced. Very probably if such effects were observed they would have been mentioned. The first one of our subjects who received intravenous uric acid, 15 mg. per kilo of body weight, as the monolithium urate in distilled water, complained of a headache, had a slight fever, and was nauseated. This solution was not isotonic and it was considered a possibility that the reaction was due to hemolysis, so in all other injections the solution was made isotonic with glucose. There were no more symptomatic effects. It is difficult to say whether tonicity was a factor here or not or whether the one case was merely accidental. However, it might be interesting to point out the recent observation of Rowntree (10) that distilled water slowly injected intravenously killed a rabbit within 10 minutes after receiving 25 cc.

The results on the recovery of the injected uric acid agree with those of Bürger (5), Griesbach (6), and Thannhauser and Weinschenk (7) and warrant the conclusion that considerable uricolysis goes on in the human body. This statement can only be made on the provisional basis that part of the uric acid is not eliminated through other channels as by means of sweat or feces. Negative evidence upon the latter would not mean very much inasmuch as bacterial decomposition probably takes place. Although traces of uric acid have been found in the sweat, it has always been assumed that the amount eliminated in this way is

	Dietary mg.	3 hrs. after. mg.	Differ- ences. mg.	Vol- ume. cc.	Liter. mg.	Total. mg.	Per hr. mg.	Recovery.	
								mg.	per cent
Case 1. T. M., male, age 39, weight 54.4 kg. 710 mg. uric acid injected. Mitral and aortic insufficiency was de- compensated wk. before.	3.71	4.88	1.17	24 hrs. before	188	107	4.5	258	36.3
				1 hr. after.	570	51	51.0		
				2 hrs. " 1st.	200	268	41.5		
				21 " " 3rd.	310	281	11.0		
				24 " " ; total.	1,500 2,010	365			
Case 2. A. B., female, age 50, weight 57.7 kg. 750 mg. uric acid injected. Polyuria, nocturia. Urea N 18.6, blood sugar 70 mg.	3.80	4.19	1.10	24 hrs. before.	170	408	16.9	465	62.0
				1 hr. after.	680	124	124.0		
				2 hrs. " 1st.	450	95	47.7		
				21 " " 3rd.	1,370	648	30.9		
				24 " " ; total.	2,450	868			
Case 3. O. H., male, age 11, weight 40.4 kg. 528 mg. uric acid injected. Apparent re- covery from acute nephritis. See Case 1, renal disease.	3.68	6.48	2.80	24 hrs. before.	181	225	10.1	255	48.4
				1 hr. after.	150	31	31.0		
				2 hrs. " 1st.	120	322	19.0		
				21 " " 3rd.	1,240	410	17.1		
				24 " " ; total.	1,510	480			
Case 4. L. H., female, age 23, weight 55.2 kg. 721 mg. uric acid injected. Preclamptic toxemia, albumin ++++, blood pressure 200/120.	3.64	7.32	3.78	24 hrs. before.	118	144	6.0	249	34.9
				1 hr. after.	1,700	108	54.0		
				2 hrs. " 1st.	490	65	32.5		
				21 " " 3rd.	210	220	10.5		
				24 " " ; total.	1,110 1,800	393			
Case 5. K. J., female, age 34, weight 54.4 kg. 710 mg. uric acid injected. Post parturi- tion, hypertension, phenol- sulfonaphthalein 32 per cent for 12 hrs. Urea N 12.1, blood sugar 70 mg.	3.16	4.88	1.72	24 hrs. before.	158	224	9.3	255	35.7
				1 hr. after.	1,400	47	14.2		
				2 hrs. " 1st.	230	28	19.3		
				21 " " 3rd.	110	405			
				24 " " ; total.	2,130 2,470	479			

Since uric acid cannot be quantitatively recovered from the urine and since the percentage recovery varies, it introduces an unknown error in its use as a measure of renal insufficiency. In spite of this objection our results show that the intravenous use of uric acid may be helpful in certain renal cases when other tests are negative. It perhaps is unnecessary to say that only a very extensive study of a large number of cases will determine the value of such a test. That much is still to be known about uric acid elimination before we can intelligently discuss its retention or destruction was recently demonstrated by Lennox (11) when he showed a marked increase of the uric acid level in the blood without comparable increase in elimination upon prolonged starvation. He points out that this may possibly be due to renal involvement although other signs of such a complication were lacking. He cites that the increase of blood uric acid may be the first sign of nephritis. His suggestion that the hyperuricacidemia reported in cases of cancer, hypertension, thermic fever, and methyl poisoning may be due to a resulting nephritis certainly is a tenable one.

Several recent attempts to localize the elimination of various substances in different parts of the excretory mechanism of the kidney are interesting in this connection and may explain the retention of certain substances in certain cases of nephritis, while in other cases the same substances may be normal and others retained. O'Connor and Conway (12) believe, after studying the localization of excretion, that uric acid is excreted in the lower part of the second convoluted tubule. It is possible that a special study of various substances in regard to retention may offer more definite knowledge as to where the pathology, if localized, exists in renal damage.

CONCLUSIONS.

1. A preliminary investigation showed that uric acid ingested by mouth did not appreciably raise the blood uric acid level or increase its excretion in the urine.

2. Only about one-half of the uric acid injected intravenously can be recovered in the urine. The amount recoverable varies with different individuals. Uricolysis is assumed.

3. The amount of uric acid retained in the blood 2 hours after injection and the rapidity of its elimination in the urine may be of value in determining renal insufficiency.

4. Variability of destruction and errors in metabolism, as gout, must be taken into consideration in the interpretation of retention of injected uric acid.

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STUDIES ON THE THEORY OF DIABETES.

IX. SUGAR EXCRETION CURVES IN DOGS UNDER INTRAVENOUS INJECTION OF GLUCOSE AT LOWER RATES.

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(Received for publication, April 8, 1924.)

In the last paper of this series (1) which appeared in 1917, experiments were described in which glucose was injected into dogs intravenously at different rates sustained constantly for periods of $\frac{1}{2}$ to 1 or several hours. The paper dealt with the effects of injections at rates rising from 0.1 to 2.0 gm. per kilo per hour. When these experiments were performed, there was no accurate method available for the estimation of such small quantities of sugar as occur in the normal urine. The best method known to us was that of Bang and Bohmannson (2), which had been used in the routine of phlorhizin experiments reported earlier. By means of this method, the attempt had been made to plot curves of urinary sugar excretion under the influence of glucose supplies rising from the basal or fasting level by steps of 0.1 gm. per kilo per hour until a gross glycosuria, detectable by the ordinary qualitative tests, appeared, but the method, in our hands, did not permit the plotting of satisfactory curves. Later, Benedict and Osterberg (3) reported a new and superior method for the quantitative estimation of the sugar in normal urine; and Folin and Berglund (4) subsequently reported another. With these methods available, it seemed advisable to repeat the earlier experiments and again attempt to plot the curve of sugar excretion as it rose from the fasting level under the influence of intravenous injections of glucose at gradually ascending rates.

The experiments were divided into two groups. Those of the first were designed mainly to ascertain the effects of intravenous

injection at a series of rates (lying between 0 and 2.0 gm. per kilo per hour) on the rate of total sugar excretion in the urine (without distinguishing between the fermentable and non-fermentable fractions). The results of these experiments are described here. Other experiments in which further observations were made of the blood sugar percentages, of the relationships between these and the urinary sugar, and of the effects of more or less water in the solution injected will be reported in another paper.

Technique of Experiments.

Dogs were used exclusively. Injections were made into a leg vein (saphenous, popliteal) by means of a volumetric pump as previously described (5). The purpose was to determine the rate of sugar excretion for each animal during fasting and under the influence of uniformly sustained injections at each of several selected rates. It had been observed repeatedly that a given animal receiving glucose by vein at a constant rate for several hours might show a relatively high excretion for the first half hour or hour, after which it settled to a lower level, and then tended to remain virtually constant for some time. It was also found that if a given animal received glucose injections at a series of ascending rates instituted one after another in the course of a single continuous experiment lasting 8 hours or more, the animal might excrete less sugar under the influence of the rate of injection instituted last than it would if injected at this rate first, or immediately after 18 hours of fasting. It seemed desirable, therefore, in testing the responses of a given dog to injections at different rates to prepare the animal by 18 to 20 hours of fasting, and inject the glucose for several hours at one rate only on a single day; then to allow several days to elapse, during which the animal lived under ordinary laboratory conditions, then to inaugurate a second period of fasting followed by injection at a second rate, and so on.

The glucose was injected in the form of 19.8 to 20.3 per cent solutions,¹ and the injections were continued for exactly 4 hours

¹ Corn Products Refining Company's c.p. glucose was used in all this work. It was dissolved in triple distilled water, to make about a 20 per cent solution, and was sterilized by autoclave. The glucose concentration was determined by polariscope directly before injection.

at each rate. The total excretion for 4 hours divided by the weight of the dog in kilos times 10 was taken as the excretion per 4 hours per 10 kilos of body weight for the individual animal. The figures obtained for each animal under different injection rates were compiled to give the curves shown. The urine was collected continuously by catheter and the bladder irrigated at the close of the periods, each sample being brought to a standard volume. These were tested qualitatively by means of Haines' and Benedict's solutions and quantitatively by the Benedict and Osterberg method.

Comment.

The dogs used in these experiments excreted, after fasting 18 to 20 hours, as little as 6 mg. and as much as 65 mg. per 10 kilos per 4 hours of total sugar as estimated by the Benedict and Osterberg method, corresponding to 36 and 390 mg. per 10 kilos per day, respectively. But the great majority of the observations fell between 17 and 35 mg. per 10 kilos per 4 hours, and the average of all was 23 mg., corresponding to 138 mg. per 10 kilos per 24 hours (which would amount to 690 mg. per 50 kilos per day). Benedict and Osterberg (6) observed in the case of a fasting dog, weighing about 18 kilos, excretions of 8.2 mg. of sugar per hour, which is equivalent to 109.2 mg. per 10 kilos per day, in fair agreement with these results.

The rate of sugar excretion in the two dogs (Nos. I and V), which received glucose by vein at 0.3 gm. per kilo per hour, was appreciably higher than the fasting rate; thus, in Dog I it rose from a fasting excretion of 23 mg. to 60 mg. and in Dog V from 12 to 64 mg. per 10 kilos per 4 hours, roughly 2.5 and 5 times the basal output, respectively. The absolute increases in the two cases were more nearly the same than the percentage elevations, but owing to the exceptionally low fasting output in the case of Dog V, this increase accounted in that case for a higher percentage rise. Injection at the 0.6 gm. rate in the case of these two dogs (Nos. I and V) caused a further elevation in the excretion to 87 mg. per 10 kilos per 4 hours in both instances. The 0.6 gm. rate in the other dogs, which were not observed at the 0.3 gm. rate, caused elevations as follows: Dog II (fasting excretion 28 mg.) to 129 mg.; Dog III (fasting 26 mg.) to 117

mg.; Dog IV (fasting 23 mg.) to 92 mg.; and so on. Thus, injections of glucose at rates from 0 up to and including 0.6 gm. per kilo per hour resulted in total increases of the sugar excretion amounting to less than five times the basal outputs. As the injections were increased above the 0.6 gm. rate a point was

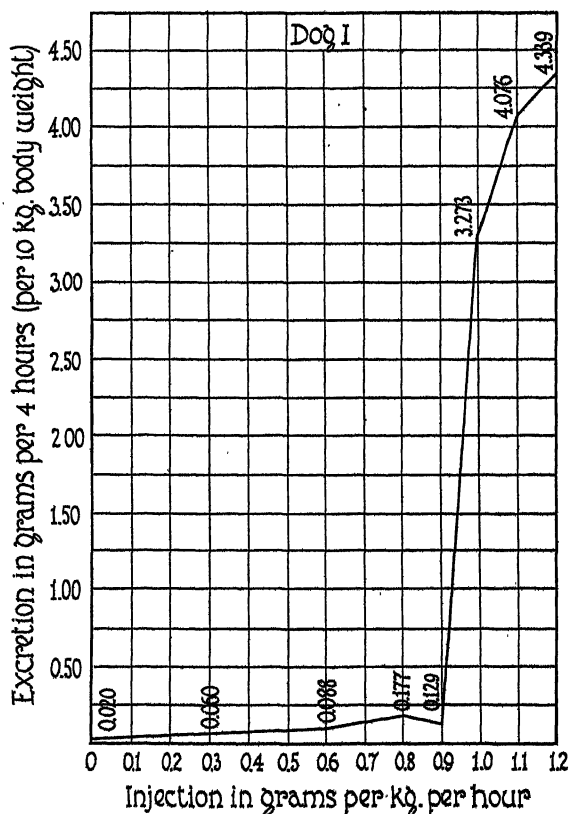


FIG. 1.

finally reached, in all cases except one, at which a further acceleration of the injection rate by 0.1 gm. per kilo per hour caused a sudden increase of the sugar excretion out of proportion to the total rise caused by all increases of the injection rate up to that point. Thus in Dog I, which in fasting excreted 18 to 31 mg. per 10 kilos per 4 hours, the highest excretion with any

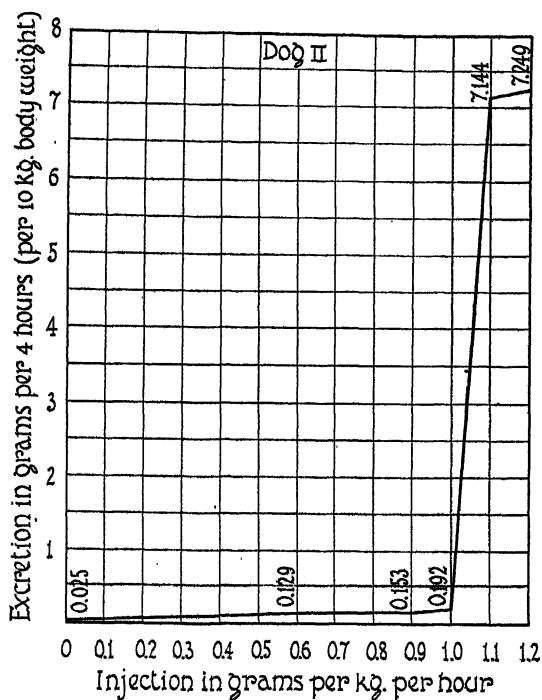


FIG. 2.

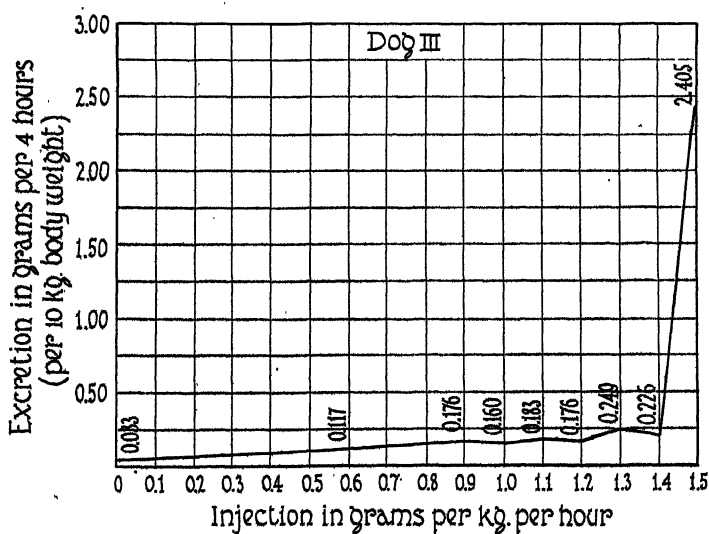


FIG. 3

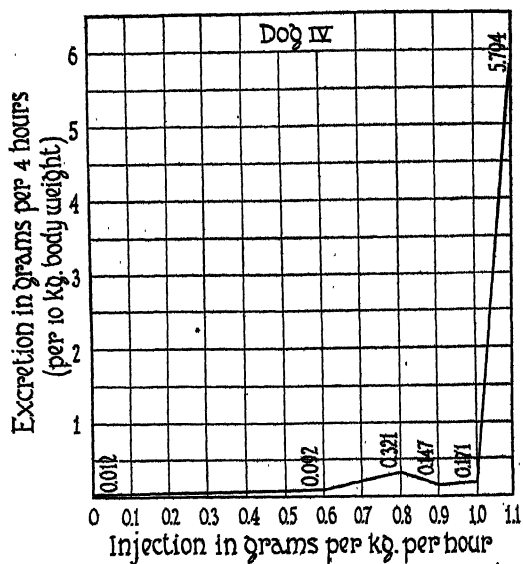


FIG. 4.

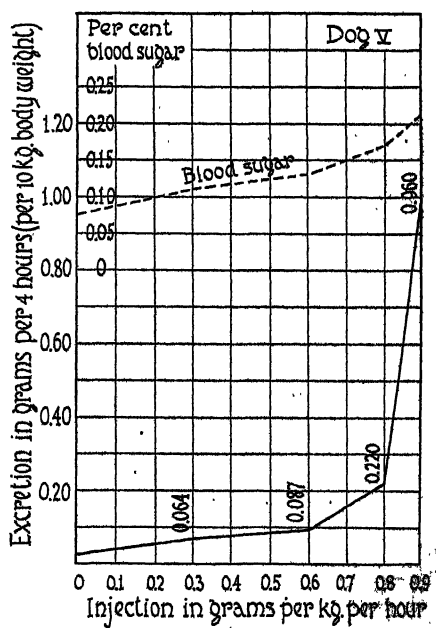


FIG. 5.

injection rate below 1.0 gm. per kilo per hour was 177 mg. or 5.7 times the exceptionally low fasting excretion shown by that dog during the control period. With the 1.0 gm. injection rate the excretion rose to 3,273 mg. or 105 times the fasting excretion. This critical phenomenon occurred in Dog I with the injection rate at 0.9; in Dog II at 1.0; in Dog III at 1.4; in Dog IV at 1.0;

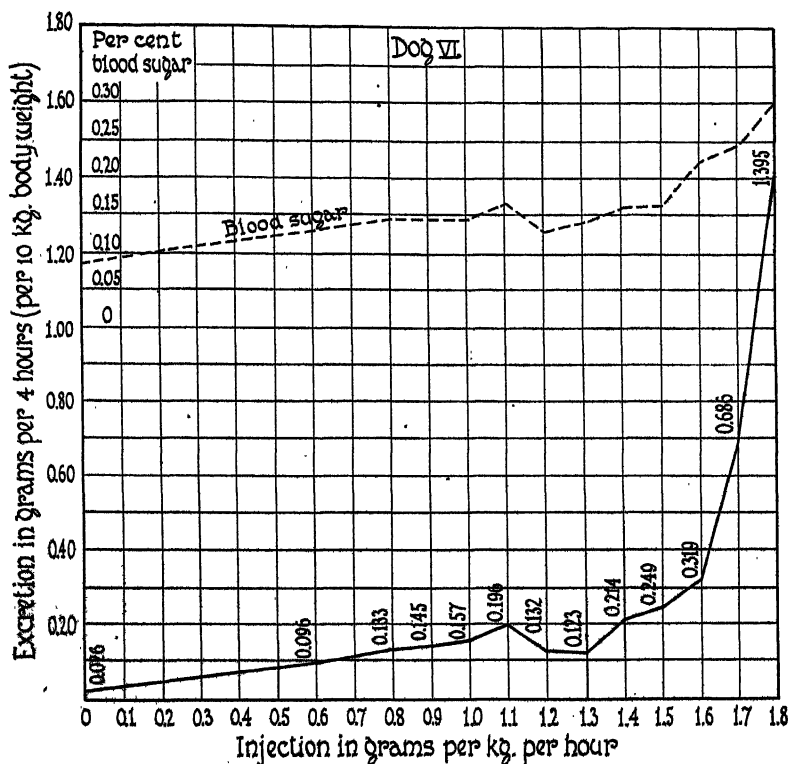


FIG. 6.

and in Dog V at 0.8 gm. per kilo per hour. In Dog VI there was no clearly definable critical point; the excretion rising gradually as the injection rate rose to 1.1, then falling somewhat, then rising again. But at 1.4 even in the case of this dog the excretion ascended steeply and broke upward disproportionately at 1.7.

Experiments of the same general character as these had previously been carried out here with injection rates rising progres-

sively above the highest reported in this series in order to study the subsequent behavior of the curves of excretion; but in those studies another method was used for estimating the sugar, so that the lower ends of the older curves could hardly be expected to approximate exactly the upper ends of the new ones. However, it may be said that with rates of injection rising above 2.0 gm. per kilo per hour the curve of excretion, although observed to rise progressively, has not continued to rise as steeply as in the critical periods of the present experiments. In other words, as the rate of glucose injection rises from 0, there is at first a slow rise of excretion, then a steep upward bend or break, then a continued rising tendency not so steep as that seen in the critical stage. In Dog I the curve broke sharply upward between 0.9 and 1.0, then rose more slowly with an advance of the rate from 1.0 to 1.1 and 1.2, and a similar phenomenon is shown in the chart of Dog II.

As to the interpretation of these steep accelerations of the rate of excretion occurring when, but not before, the injection rate rises above a certain value, judgment is reserved. Whether the break is referable to an excretory phenomenon or whether it implies that the power to utilize glucose is temporarily overtaxed at this point after which, under the stimulus of an excess of sugar, it rises to a higher level after the manner of heart action in the phenomenon of "second wind," or whether it is due to some other physiological event, the experiments would not decide. They appear to indicate, however, that with glucose supplies rising gradually above the fasting endogenous level a critical physiological event takes place when, and not before, the supply rate exceeds a certain limit sharply definable for that case.

Using the qualitative test solutions, we have not failed to obtain positive reactions after the critical point has been reached. The urines of the precritical periods, however, have often contained enough sugar to give faint positive reactions when the tests have been made under the standard conditions adopted by us; namely, 2 cc. of urine in 5 cc. of reagent.

Folin and Berglund (7) working with alimentary administrations of various carbohydrates including glucose, in man, concluded that "the ingestion of pure glucose (up to 200 gm.) does

not raise the level of the blood sugar above the threshold in normal persons, and no glycosuria is obtained." In the present experiments, working with dogs and intravenous administration, small doses of glucose have regularly increased the elimination of total sugar. This finding, however, is not necessarily in conflict with the above; first, because it was not absolutely proved in the present experiments that the observed increases in sugar excretion preceding the critical elevations were due to glucose in the urine, although it might seem plausible to assume that they were, provided the glucose injected was strictly pure; secondly, because in the present experiments dogs were used instead of men and the administrations were by *peripheral vein* and not into the portal circuit as in Folin and Berglund's work. Working with human cases (8) and diets in which the glucose-yielding power² was increasing, we also have failed in a few cases to note increases of sugar excretion above the fasting levels, but in the majority of such cases of which we have records increases have been observed. In extended experiments on three animals in which they studied the effects of subcutaneously injected glucose on the urinary sugar output in the dog, Benedict and Osterberg (9) found that following the injection of 0.4 gm. of glucose per kilo of body weight and upward there is a definite increase in the total sugar output. Their injections, however, were given following the administration of standard meals, and with no certain means of estimating the exact rates of absorption of the subcutaneous doses or the rate at which glucose was supplied by the feedings, a quantitative comparison of their results with those here reported is not possible.

The present experiments were designed mainly to ascertain whether or not under gradually rising glucose supplies there is for any particular individual a critical rate of supply which when exceeded leads to a sudden acceleration of the output of sugar in the urine, or whether the transition from the normal sugar excretion to a gross glycosuria is a gradual process, as suggested by Benedict, Osterberg, and Neuwirth (10). That it may possibly be gradual in some cases is not disproved, but our experiments indicate that in most cases there is an abrupt phenomenon. As

² Calculating the glucose-yielding power of a mixture metabolized as the carbohydrate plus the protein times 0.58 plus the fat times 0.10.

to whether the slight precritical increases of the total sugar excretion observed in these experiments were due wholly to glucose or partly to the presence of unknown impurities in the glucose injected is a question not bearing essentially on the main theme of the paper.

CONCLUSIONS.

The results essentially confirm the conception that there is for any individual animal a certain maximum rate at which glucose may be introduced continuously into the systemic circulation without the occurrence of gross glycosuria, and that when this rate is overstepped a sharp increase of the sugar output usually occurs. The ordinary qualitative tests for sugar in the urine, if not too refined, yield as a rule negative reactions before and almost invariably positive tests after the critical point has been reached.

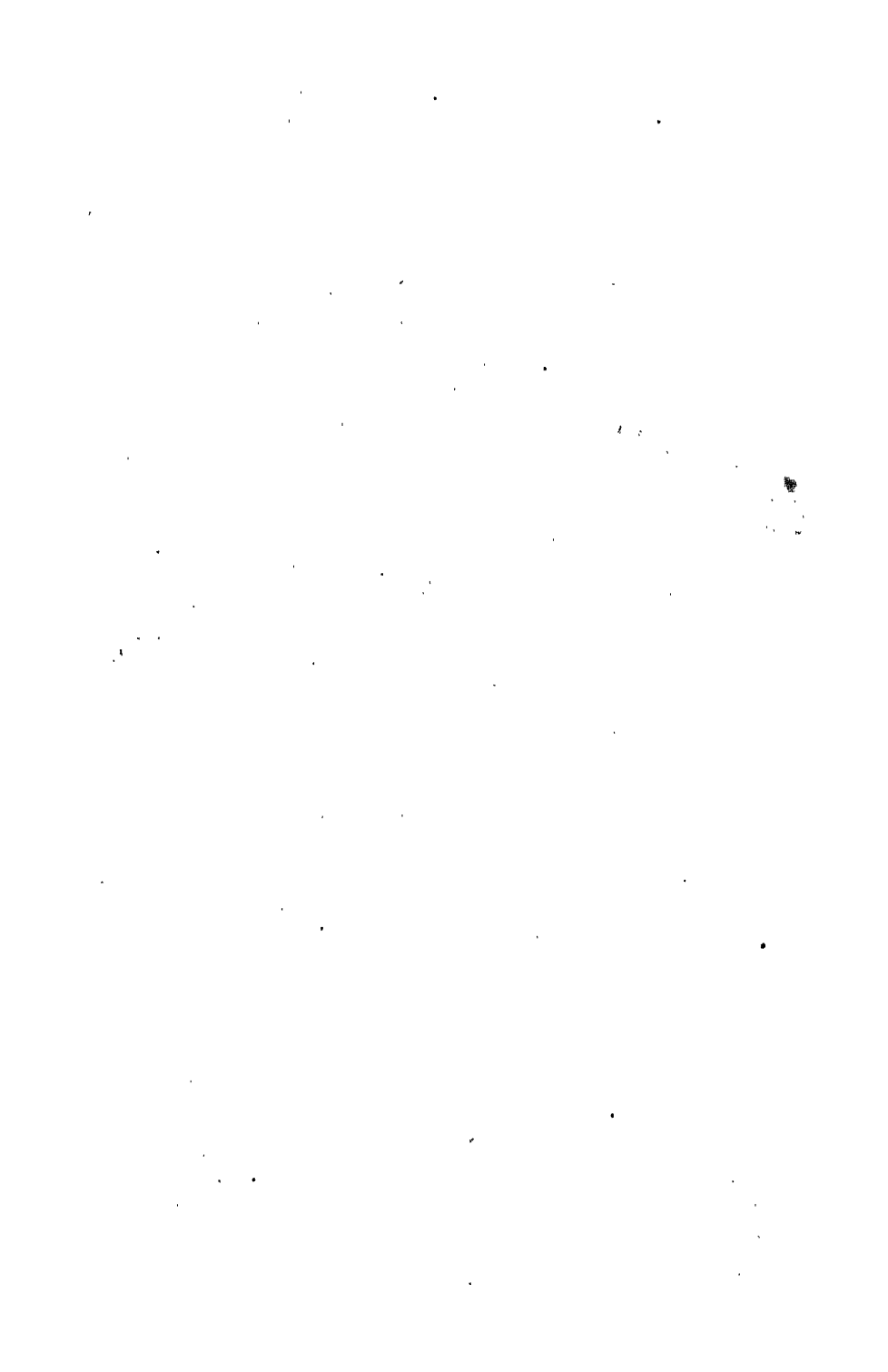
Gradually increasing the rate of injection of glucose into the systemic circulation of normal fasting dogs from 0 to 0.8 gm. or thereabouts per kilo per hour causes a measurable but relatively slight progressive increase in the rate of sugar elimination in the urine until for any individual a certain critical rate is instituted when there usually occurs a sharp acceleration of the sugar excretion out of proportion to any that occurs before. Thus in the cases of the five dogs that showed a sharply definable critical point, increasing the rate of intravenous injection of glucose from 0 up to that critical point has led to aggregate increases of the sugar elimination averaging 4.1 mg. per kilo per hour above the basal or fasting levels; whereas when the critical rate (0.8, 0.9, 1.0, 1.0 gm. per kilo per hour, respectively, in 4 dogs and 1.4 gm. in the fifth) was overstepped the aggregate excretion above basal averaged 97.3 mg. per kilo per hour, or 24 times as much. The effect of increasing the injection rate by 0.1 gm. per kilo per hour in the critical stage was 225 times as great on the average as in the precritical stages.

The compiled curves representing graphically the total excretion of sugar by 4 hour periods per 10 kilos of body weight for individual dogs receiving glucose by vein at rates ascending from 0 to the critical level show therefore in most instances a first portion that inclines upward relatively little from the start

zontal followed by a second portion in which the curve shows a steep upward bend or break. Further gradual increases in the rate of injection affect the subsequent course of the curve in a manner to be described in a later report.

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THE METABOLISM OF SOME PYRIMIDINES.*

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INTRODUCTION.

The demonstration by Johnson and Baudisch (1) that thymine is readily oxidized at ordinary temperature with such a mild oxidizing reagent as ferrous hydroxide and peroxide renewed our interest in a study of the metabolic behavior of this pyrimidine and also uracil to determine whether an analogous destruction of these substances might not occur in the animal organism. The fate of the pyrimidines has been a problematical question since their isolation as hydrolytic derivatives of nucleic acid 30 years ago. Inasmuch as pyrimidines may be recovered from the urine unchanged after they are fed in fairly large amounts in a free state or in the nucleoside or nucleotide combination, but not when fed as a constituent of nucleic acid, the hypothesis is current that in normal metabolism these substances must be decomposed before the nucleic acid molecule has suffered cleavage to the mononucleotide state. In other words, the animal organism is able to destroy the pyrimidines as long as they exist as an integral part of the tetranucleotide molecule, but this is no longer possible when the compounds are split into mononucleotides or farther into nucleosides—a transformation which is not supposed to alter in any way the pyrimidine constituent existing therein.

* The data of this paper are taken from a dissertation presented by H. J. Deuel, Jr., to the faculty of the Graduate School of Yale University, 1923, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

A preliminary account of some of the experiments was published by Deuel and Mendel on the metabolism of thymine (Deuel, H. J., Jr., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, 1922-23, xx, 237).

HISTORICAL.

The physiological behavior of the pyrimidine group was first studied by Wöhler and Frerichs (2) who fed 6 gm. doses of alloxantin to men. None of this compound could be recovered from the urine and these authors suggest a breaking down of this substance to urea since the latter occurred in increased amounts in the urine following the administration of this pyrimidine. Koehne (3) confirmed these results since he was unable to detect either alloxan or alloxantin after feeding 8 gm. doses of these pyrimidines.

Steudel (4) investigated the behavior of a number of pyrimidines by feeding 1 gm. doses of the substances under study to a bitch. Thymine, uracil, isobarbituric acid, and isodialuric acid were apparently completely destroyed since none of these compounds could be recovered from the urine collected in the period subsequent to their administration, although 4-methyl uracil, which differs from thymine only in the position of the methyl group, and 5-nitrouracil were found to be excreted. Steudel (5) later reported that hydrouracil and imido methyl uracil are destroyed by the dog when administered *per os*, while 4-methyl sulfouracil escapes physiological conversion.

Sweet and Levene (6), using an Eck fistula dog, showed that thymine when administered orally in a dose of 6 gm. was more than half excreted unchanged. Mendel and Myers (7) subsequently confirmed this result, the latter investigators finding that the administration of 1 or 2 gm. of thymine to rabbits or of 3 gm. to a dog resulted in its partial excretion. It should be noted, however, that in two of their experiments a marked rise in urea nitrogen resulted on the day of thymine ingestion, thus indicating the possibility of a partial physiological destruction of the pyrimidine. The above investigators obtained like results after feeding uracil. When 1.5 gm. were given to a rabbit and a somewhat larger amount to a dog, a partial excretion of this substance followed, but a concomitant rise in urea excretion was noted. Cytosine was also partially excreted by the dog after its oral administration; the protocols of Mendel and Myers do not demonstrate that a conversion of this substance to urea took place. The fate of this amino pyrimidine in this respect we should not expect to differ from that of uracil since its amino group is detached with great ease, giving rise to uracil.

A number of investigations have been carried out to determine the effect of nucleic acid feeding on the nitrogen partition of the urine, but in only a few instances have attempts been made to determine whether free pyrimidines are excreted as a result of the hydrolytic cleavage of the nucleic acid molecule in the animal organism. Frank and Schittenhelm (8) showed that a large increase in urinary urea nitrogen was coincident with the ingestion of 10 gm. of yeast nucleic acid by a man who was on a constant fixed diet, thus illustrating that when the pyrimidines are in the normal nucleic acid combination they are largely broken down to urea. Neither Sweet and Levene (6) after feeding 10 gm. of thymus nucleic acid nor Mendel and

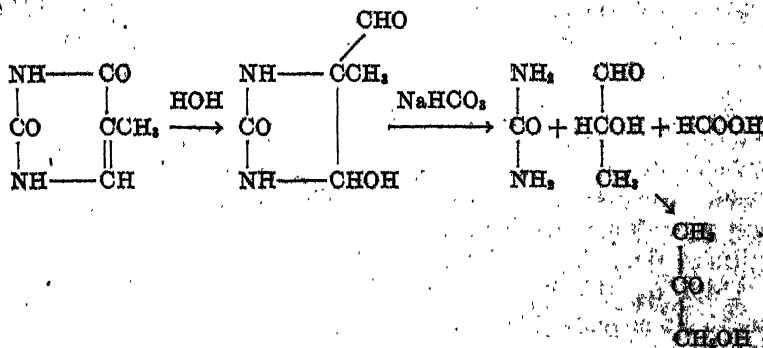
Myers (7) nor Wilson (9) after the administration of the same quantity of yeast nucleic acid could detect any pyrimidines in the urine.

Wilson (10) has likewise fed a nucleotide, uridine phosphoric acid, and a nucleoside, uridine, to rabbits—both compounds causing an excretion of free uracil but likewise a concomitant rise in urea excretion. Wilson has interpreted these data as suggestive of the hypothesis that the pyrimidines are destroyed while still in the nucleic acid combination, but are incapable of such transformations after the nucleic acid molecule has undergone partial or complete hydrolysis.

In the more strictly chemical studies on the behavior of the pyrimidines the recent work of Johnson and Baudisch and collaborators is of interest from a physiological standpoint. Johnson and Baudisch (1) have recently shown that the supposedly highly resistant pyrimidine ring is readily destroyed by the action of ferrous hydroxide and peroxide at room temperature—thymine, for example, yielding under such treatment urea, acetole, pyruvic acid, and formic acid.¹ Uracil, 5-ethyl uracil, 4-methyl uracil, cytosine, and thymine have been shown by Bass and Baudisch (12) to be decomposed to give urea as one of their end-products under the influence of iodine solution while thymine undergoes a slight conversion into urea when illuminated by the quartz mercury arc light especially in the presence of ferrous sulfate (Bass (13)). In view of the marked ease with which pyrimidines are decomposed by very mild chemical treatment, their behavior under physiological conditions assumes added interest.

The investigation herein described was undertaken to determine whether in the course of normal nucleic acid metabolism, the pyrimidines might not be set free and subsequently suffer further destruction as such. In the first place in order to determine whether pyrimidines might occur in small quantity in normal

¹ Baudisch and Bass (11) have suggested the following mechanism for this decomposition:



urine, an attempt was made to isolate them from a large quantity of normal human urine. Secondly, the question was investigated whether a large dose of a pyrimidine, which is known to be partially excreted unchanged, might not cause a concomitant rise in urinary urea excretion which would indicate the possibility for the organism to split limited quantities of the pyrimidines. Next, a study was made to determine whether the free pyrimidines would suffer complete decomposition if administered in very small amounts over a long period of time. Finally, very large quantities of nucleic acid were fed to a dog in an attempt to overflow the organism with pyrimidines and thus bring about an excretion thereof if these should be intermediates in nucleic acid metabolism.

For these investigations dogs were fed on diets containing a fixed amount of nitrogen according to the method recently described by Cowgill (14). Analyses were made as follows: total nitrogen by the Kjeldahl method, urea by the Marshall method, sulfates according to the procedure outlined by Folin, and pyrimidine recovery by precipitation with Hopkins' reagent as described by Mendel and Myers (7). Thymine and uracil were synthesized by the methods developed by Wheeler and Johnson while thymus nucleic acid of good quality was prepared in sufficient quantity for the experiment by the method of Levene (15).

EXPERIMENTAL.

Pyrimidines in Normal Urine.—No investigation is on record, as far as known to the author, in which an attempt has been made to recover pyrimidines from large amounts of normal urine. It might readily be supposed that small amounts of pyrimidines under usual conditions of diet are constantly being excreted, their relatively high solubility making their detection much more difficult than that of the purines. This assumption seemed possible in view of the fact that by application of the Johnson-Baudisch reaction to urine (16) to detect thymine, a positive result was always obtained which was caused by some other constituent than the small amount of glucose which was present.

Urine for this investigation was obtained in amounts of 10 to 15 liters daily from the surgical wards of the New Haven Hospital from patients who had been on the usual hospital diet.

The urine as collected was preserved with toluene and was received from the hospital daily. A total of 150 liters was concentrated by evaporating it in large evaporating dishes to a volume of about 15 liters, the resulting precipitate being filtered off and discarded and the filtrate concentrated to 3,500 cc. After the removal of a large amount of urea which had separated out, the filtrate was made up to 5 liters and an equal volume of saturated mercuric chloride solution was added, the solution made alkaline with sodium hydroxide—the general plan of Mendel and Myers (7) being used in an attempt to isolate thymine from the urine. From this amount of normal urine, it was impossible to isolate any thymine or cytosine, thus indicating that if any pyrimidines do escape physiological oxidation to be excreted into the urine as such, the amount must be exceedingly small. Under usual conditions of diet, therefore, it may be concluded that the pyrimidines obtained from the metabolism of endogenous and exogenous nucleic acid, undergo complete physiological conversion with the result that none escapes into the urine unchanged.

Experiments with Thymine.—In the first series of experiments thymine was administered as such to dogs in a single large dose and the amount of extra urea resulting therefrom noted; any pyrimidine which was excreted in the urine was isolated and weighed. Nine experiments were carried out on two dogs in which 1 to 4 gm. of thymine were administered. In six of these experiments an unmistakable rise in urea nitrogen occurred on the day of thymine ingestion while the results of the other three tests are inconclusive. Table I shows one of the experiments which demonstrates the increased urea excretion coincident with the day of thymine ingestion. On account of the length of the tables, other results are not included here.

From the urine on the day of thymine ingestion, 0.096 gm. of impure thymine was recovered which gave a low nitrogen value—20.94 per cent instead of the theoretical 22.22 per cent. Table II gives the results of another experiment in which a larger amount of thymine was fed. The urea nitrogen does not show such a marked increase as in the former test. 0.874 gm. of pure thymine was recovered from the urine of the day of thymine feeding which gave a nitrogen content of 21.79 per cent instead of the theoretical value of 22.22 per cent.

The increase in urea excretion following the ingestion of thymine was a real augmentation and could not be ascribed to any

TABLE I.
Experiment on Thymine Metabolism.
Dog H. Weight 5.35 kilos.

Dec.	Volume of urine.	Nitrogen.			Sulfur.	Thymine fed.
		Total.	Urea.	NH ₃		
1922	cc.	gm.	gm.	gm.	gm.	
21-22	325*	2.66	2.20	0.07	0.088	
22-23	225*	2.79	2.16	0.17	0.100	
23-24	120	2.69	2.07	0.16	0.086	
24-25	90	2.70	2.31	0.04	0.084	
25-26	178	3.00	2.53	0.11	0.079	1.5 gm. in 50 cc. solution N ₂ = 0.33 gm.
26-27	90	2.56	2.07	0.13	0.068	
27-28	80	2.62	2.11	0.13	0.084	
28-29	100	2.83	2.19	0.19	0.086	

* The large quantities of urine in the first 3 days of the experiment represent the effect of ingestion of 3 gm. of thymine on the day previous to the start of this experiment.

TABLE II.
Experiment on Thymine Metabolism.
Dog H. Weight 5.3 kilos.

Dec.	Volume of urine.	Nitrogen.			Thymine fed.
		Total.	Urea.	NH ₃	
1922	cc.	gm.	gm.	gm.	
16-17	130	2.84	2.30	0.17	
17-18	90	2.77	2.20	0.16	
18-19					Urine lost.
19-20	90	2.85	2.28	0.17	
20-21	450	3.46	2.39	0.04	3 gm. Na thymine given in 50 cc. solution N ₂ = 0.66 gm.
21-22	325	2.66	2.20	0.07	
22-23	225	2.79	2.16	0.17	
23-24	120	2.69	2.07	0.16	

of the causes enumerated below which might be conceived as responsible for it. An increased urea excretion might be ascribed to an augmented protein catabolism brought about in some way

by the action of thymine although such an effect is extremely improbable. However, were the protein metabolism greater, the excretion of sulfur as well as of urea nitrogen should be increased. The excretion of total sulfates remained constant in the experiments which showed a greater excretion of urea nitrogen.

The diuresis which usually accompanies the ingestion of thymine might readily be supposed to wash out an added quantity of urea since it is known that when a very large amount of water is administered there is a temporary increase in urea output. However, control experiments, the protocols of which are not included, did not demonstrate that a diuresis to the extent caused by thymine influenced the urea excretion. Moreover, in some experiments which showed an increased urea output following the feeding of this pyrimidine no diuresis occurred.

In order to be certain that urease employed in the analytical procedures did not act on thymine as well as on urea, the urea content of a sample of urine containing a known amount of thymine and a sample of the same urine without the pyrimidine were determined and found to be identical—a result which proves that the urease method can be satisfactorily applied to urines containing the pyrimidine thymine.

The administration of the sodium salt of thymine which is somewhat alkaline often resulted in a decline in the ammonia content of the urine. That the increased urea excretion coincident with the thymine administration was not a result of this ammonia nitrogen thus made available for urea synthesis seems likely from the experiments of McCollum and Hoagland (17); moreover, certain experiments did not show this decline in ammonia nitrogen which at the same time showed the increase in urea nitrogen excretion.

Lastly, the administration of thymine did not render the urine abnormal; after the administration of this pyrimidine, routine tests for sugar and albumin were always a negative.

On the basis of these experiments it seems fair to assume that thymine can be decomposed to some extent in the animal body when this pyrimidine is administered as such. In the second series of experiments which follow, a study was made to determine whether the animal organism could completely destroy small quantities of thymine. For these experiments quantities of

thymine which, when fed in a single dose, were excreted to a considerable extent, were fed in small divided doses over a comparatively long period of time and an attempt was made to recover this pyrimidine from the combined urine of the experimental period. In one experiment 0.25 gm. of thymine was given daily to a dog of 5 kilos body weight over a period of 12 days. When the total amount was given to this animal in a single dose, about 1 gm. of unchanged pyrimidine appeared in the urine on the day of its administration.

From 2,614 cc. of a total volume of 2,910 cc. for the 12 days during which thymine was being administered, it was impossible to isolate a trace of this pyrimidine by the methods which had been used for the separation of this substance in the previous experiments. This experimental result would seem to indicate that the dog is able to destroy completely thymine fed in small amounts. This is further confirmed by the fact that the non-urea nitrogen for the experimental period not only did not increase but actually decreased during the period of thymine feeding. If the thymine administered in these small amounts were excreted unchanged, the non-urea nitrogen should be increased about 0.05 gm. daily during the experimental period. On the other hand, if all the nitrogen of thymine were converted to urea, the non-urea fraction for the experimental period would be unaltered. In the above experiment the daily average of the non-urea fraction for 22 days with the same animal on the diet without thymine amounted to 0.53 gm., while the mean quantity of non-urea nitrogen for the 12 days during which 0.25 gm. of thymine was administered daily was 0.50 gm. instead of 0.58 gm. which would have been expected had all the thymine been excreted unchanged.

A second experiment in which small amounts of thymine were fed over a period of days was carried out on another animal. This dog, which weighed 12 kilos, excreted 0.35 gm. of thymine after the feeding of 4 gm. by mouth in a single dose. When 0.4 gm. of thymine was fed daily over a period of 15 days, making a total of 6 gm. ingested, it was impossible to isolate any trace of this pyrimidine from 4,240 cc. of a total of 4,676 cc. of the combined urine or the experimental period.

Experiments with Uracil.—The plan of the experiments with uracil was similar to that followed with thymine, the first series

of tests being designed to study the effect of relatively large doses of this substance on the urea excretion and the second set of experiments involving the feeding of a similar quantity of uracil in small divided doses over a period of days in order to determine whether under these conditions any of this substance was excreted in the urine.

Three experiments were carried out in which uracil was administered as the sodium salt to a dog in doses varying from 0.5 to 3.0 gm. In each case a rise in urea nitrogen occurred on the day of uracil administration, the best results obtained being shown in

TABLE III.
Experiment on Uracil Metabolism.
Dog H. Weight 5.2 kilos.

Feb.	Volume of urine.	Nitrogen.			Uracil fed.
		Total.	Urea.	NH ₃	
1923	cc.	gm.	gm.	gm.	
18-19	100	3.25	2.72	0.18	
19-20	290	3.24	2.60	0.18	
20-21	80	3.08	2.51	0.15	
21-22	90	3.25	2.62	0.19	
22-23		3.23	2.64	0.16	
23-24	130	3.61	2.87	0.09	1.5 gm. of Na uracil given in 90 cc. solution N ₂ = 0.37 gm.
24-25	90	3.29	2.73	0.18	
25-26	80	3.11	2.52	0.15	

Table III. From the urine of the day of uracil feeding, 0.244 gm. of this substance was recovered which gave a strong positive qualitative test for this pyrimidine.

In the other experiments on uracil metabolism, protocols for which are not included here, a similar though not as marked an increase in urea excretion occurred coincident with the administration of uracil. When 0.5 gm. of uracil was fed, this pyrimidine was entirely destroyed as evidenced by the impossibility of obtaining even a qualitative test for it in the urine.

The control experiments for uracil are the same as those for thymine which are discussed earlier. A test was carried out to determine whether uracil might be decomposed by urease to

ammonia and in this way vitiate the urea determinations which were made by that method; urine to which a known quantity of uracil was added gave identical values for urea by the urease method with that obtained on the same urine without the addition of this pyrimidine. As in the case of thymine, the urine on the day of uracil administration showed no trace of glucose or albumin indicating that this pyrimidine did not have a pathological effect on the kidney.

In the second series of tests 0.2 gm. of uracil was fed daily to the same animal as in the previous experiment over a period of 15 days, making a total of 3 gm. No uracil could be recovered from 3,500 cc. of a total volume of 3,872 cc. for the experimental period nor could a positive qualitative test be obtained on the fraction which should include any uracil present. Moreover, the non-urea nitrogen did not change significantly during the experimental period. The average value of the non-urea fraction for 5 days before and 5 days after the uracil feeding was 0.56 gm., while the average for the days during which uracil was being fed 0.2 gm. daily was 0.57 gm. instead of 0.61 gm. which should have been the result had all the uracil been excreted unchanged.

Experiments with Nucleic Acid.—The experiments reported below were designed to demonstrate whether the pyrimidines are set free as an intermediate product in normal nucleic acid catabolism. Large quantities of nucleic acid were fed in order that the pyrimidines, if liberated, might exceed the capacity for their destruction and, therefore, result in their partial excretion.

Two experiments were carried out in which nucleic acid was fed, but the first was somewhat inconclusive since it was uncertain whether any vomitus of the animal was mixed with the urine. The protocol of these experiments is given in Table IV.

In the above experiments, 5 cc. of the urine from March 19 to 20 and 21 to 22 gave a strong positive Wheeler-Johnson test for free cytosine as did the filtrate from the decomposition of the precipitate resulting with Hopkins' reagent. The Wheeler-Johnson test for uracil and cytosine is only given by these pyrimidines when uncombined and, therefore, these urines must have contained either one or both of these pyrimidines in a free state. The amount of pyrimidine present was insufficient, however, to permit its isolation from the urine.

The urine of the day previous and following the experiments gave negative tests for cytosine. The thymus nucleic acid used, which had been prepared from the thymus glands of sheep and calves, also was free from uncombined pyrimidines as evidenced by its failure to respond to a test for free cytosine.

TABLE IV.

Experiment on Nucleic Acid Metabolism.

Dog H. Weight 5.25 kilos.

Mar.	Volume of urine.	Total nitrogen.	P ₂ O ₅	Nucleic acid fed.
1933	cc.	gm.	gm.	
15-16	100	2.74	0.16	
16-17	100	2.63	0.12	
17-18	110	2.80	0.19	
18-19	120	2.79	0.22	
19-20		3.95		50 gm. thymus nucleic acid given by stomach tube.*
20-21	300	2.89	0.59	
21-22	650	8.46†	4.88	50 gm. thymus nucleic acid given.‡
22-23		3.44	0.74	
23-24	150	2.74	0.42	

* Approximately 50 gm. of thymus nucleic acid were given by stomach tube as the sodium salt, the total volume introduced amounting to about 400 cc. A considerable amount of this was vomited and the vomitus was given again by stomach tube. This was vomited again a second and a third time; it was finally estimated that the dog retained about 20 gm. of the nucleic acid administered. The urine was collected separately from the vomitus, but it is not known whether any urine was lost in the vomitus.

† The actual amount of food administered on this day was increased so that 5.08 gm. of N₂ were fed instead of the usual 3.38 gm. from this source.

‡ 25 gm. of thymus nucleic acid were mixed with the food and fed to the dog in this way. The remaining 25 gm. were given as the sodium salt. On the preparation of this, it gelatinized to a mass resembling a stiff pudding and lumps of this were force fed. It is estimated that the dog received about 45 gm. of nucleic acid in this last experiment. There was no vomiting in the second experiment.

It would seem probable that the pyrimidines are split off under normal conditions of nucleic acid metabolism but never escape in the urine following the ingestion of ordinary amounts of this compound since the animal organism is capable of decomposing fairly large portions of them as such to urea and other undeter-

mined products. It is only when the animal organism is flooded by excessive doses of nucleic acid—a condition only met with experimentally—that sufficient pyrimidines arise to cause their excretion.

DISCUSSION.

The analytical data reported here make it seem probable that thymine and uracil in uncombined form are capable of being metabolized in the animal body so that urea results as one of their end-products. This assumption is based upon the observations that administration of uracil or thymine was usually followed by an increase in urinary urea excretion; also by the proof that quantities of these pyrimidines, which if given in a single dose would result in a comparatively large excretion of unchanged pyrimidine, were apparently destroyed when fed in a number of small divided doses. Although no experiments are available on the behavior of cytosine as such, there is no reason to suppose that this pyrimidine would act differently from thymine or uracil.

When nucleic acid is administered in sufficient amount, a slight excretion of free pyrimidine may result—a fact which would indicate that under normal conditions of metabolism nucleic acid suffers complete hydrolysis to its pyrimidine components which, in turn, when not supplied to the animal organism in too large amounts are completely broken down to urea.

This conception of pyrimidine metabolism is at variance with the present accepted ideas in the following ways:

1. Levene, Mendel and Myers, and Wilson all assume that, since after the administration of free pyrimidine in sufficient quantity some of it is excreted unchanged, we must consider that under normal conditions of nucleic acid metabolism in the animal body, the pyrimidines are destroyed before they are set free—this decomposition being assumed to take place while the pyrimidines are in the tetranucleotide combination. Although the earlier investigations have been confirmed by the experiments here reported, it does not seem necessary to postulate that because the organism is unable to utilize completely comparatively large doses of the pyrimidines it is equally unable to destroy small amounts. Even such a readily oxidizable substance as glucose is excreted when an excessive dose is administered within a short

time. Might not the excretion of pyrimidine which has been noted after the ingestion of a comparatively large amount of this substance be analogous to an alimentary glycosuria resulting after very large doses of glucose? Normally, the nucleic acid catabolism must proceed slowly so that the amount of pyrimidine set free at any moment would be many times less than that offered in the experiments reported here. The fact that the administration of a pyrimidine in much larger than physiological doses results in a partial excretion of this substance unchanged does not preclude the possibility of its complete destruction when the same compound is fed in physiological amounts.

2. Wilson assumes that since he obtained free uracil in the urine after the administration of uridine or uridine phosphoric acid, the pyrimidine must be decomposed before the nucleic acid molecule has reached the mononucleotide state—presumably while still in the tetranucleotide combination. That the animal organism could readily destroy uracil as a constituent of a tetranucleotide but not when the product is in the mononucleotide or nucleoside linkage would seem open to question were there not some striking difference in physical or chemical properties between the tetra- and mononucleotide to account for it. Such, however, is not the case. Are not these experimental data of Wilson another example of the results of flooding the animal with a substance which in small amounts might have been completely broken down? If we draw another analogy between these phenomena and alimentary glycosuria, we may conceive of uracil excretion following uridine feeding to be similar to that resulting from a very large ingestion of sucrose or other disaccharide while the relative difficulty of inducing a glycosuria after starch or dextrin ingestion might be compared with the difficulty in demonstrating pyrimidine excretion after nucleic acid feeding.

3. The fact that neither Levene, Mendel and Myers, nor Wilson have found pyrimidines present in the urine after nucleic acid feeding is another point at which the experiments here reported are at variance with the former ones mentioned. Levene fed 10 gm. of an animal nucleic acid in his experiments while Mendel and Myers and Wilson fed the same quantity of yeast nucleic acid. That these investigators have not detected free pyrimidine in the urine following the feeding of nucleic acid may be explained

on several assumptions. In the first place, it is possible that the organism might be able to decompose completely the pyrimidines were they freed from this amount of nucleic acid immediately, since the amount of each pyrimidine in 10 gm. of nucleic acid is somewhat less than 1 gm. However, the pyrimidines must be freed at a relatively slow rate and the complete destruction of the nucleic acid would probably not occur for a number of hours; the concentration of the pyrimidines offered to the organism at any time must be very small.

Secondly, we have no data on the extent of absorption of the nucleic acid. A considerable amount of it may have remained unutilized and for that reason the concentration of the residual pyrimidine actually formed would not be sufficient to cause its excretion.

However, the experiments reported here indicate that if sufficiently large amounts of nucleic acid are administered, free pyrimidine can be detected in the urine. This would seem to offer cogent evidence that the failure of Levene, Mendel and Myers and Wilson to obtain a test for the free pyrimidine after the feeding of nucleic acid was attributable to the smaller doses of this substance which these investigators fed.

SUMMARY.

When the pyrimidines, thymine and uracil, were administered in fairly large amounts (1.0 to 3.0 gm.) to dogs, a considerable quantity could be isolated from the urine. However, when the same quantities were given in small divided doses over a period of days, the animal was apparently able to utilize it for none could be detected in the combined urine of the experimental period. A detectable increase in urea usually followed the introduction of thymine or uracil which would indicate that these pyrimidines were partially metabolized to urea under these conditions by the dog.

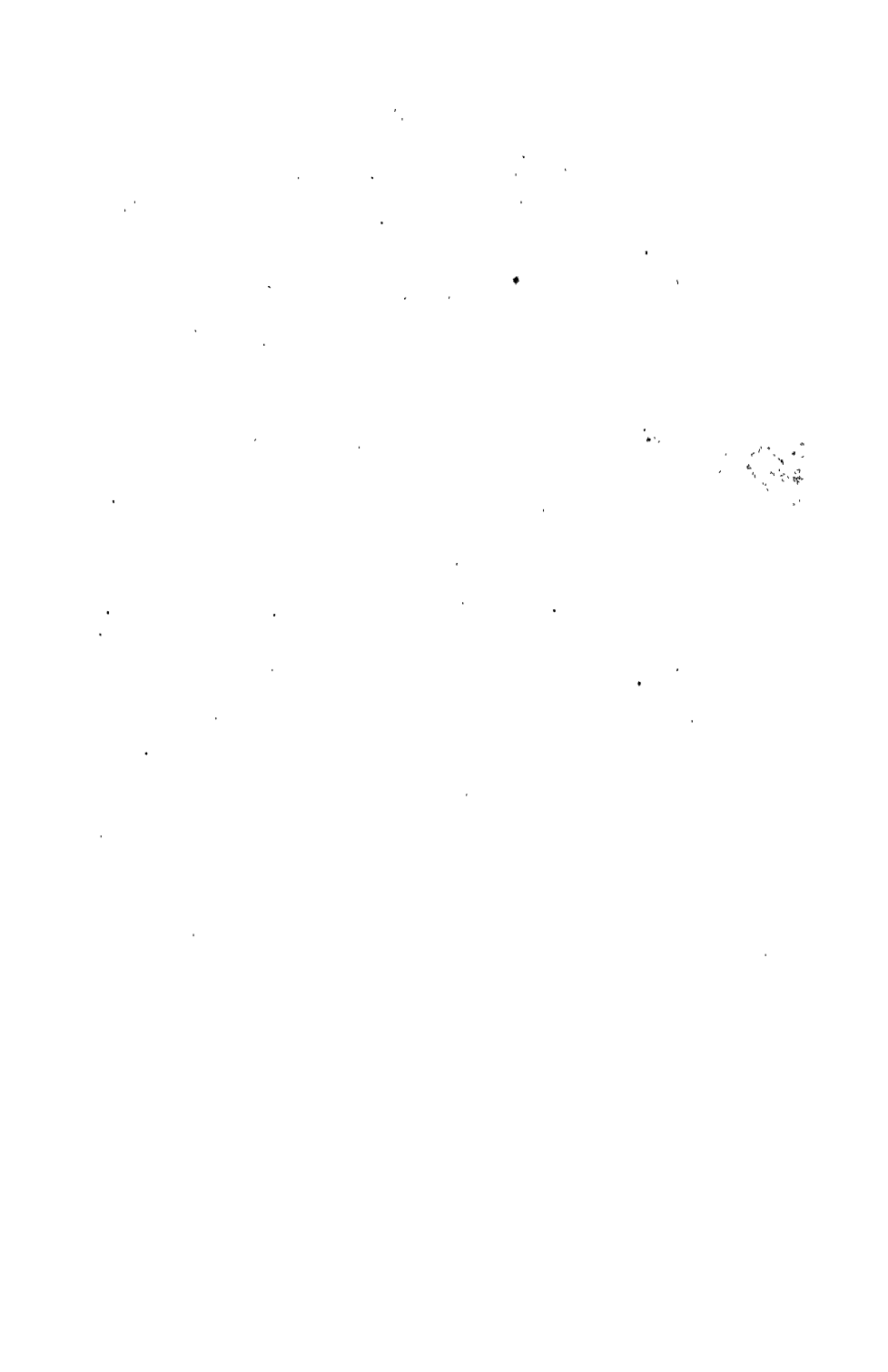
When thymus nucleic acid was fed in a 50 gm. dose to a dog it was possible to demonstrate the presence of free pyrimidine in the urine—thus suggesting that under normal conditions of nucleic acid metabolism, pyrimidines are constantly set free in an uncombined form but are not present in sufficient quantity to be excreted as such.

Under normal conditions of diet in man, pyrimidines do not escape physiological conversion since it was impossible to isolate even a trace of them in 150 liters of normal human urine.

The author gratefully acknowledges the helpful criticism of Professor Lafayette B. Mendel who suggested the problem and under whom the study was carried out.

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A COMPARISON OF THE RATE OF GLYCOLYSIS IN DIFFERENT BLOODS WITH SPECIAL REFERENCE TO DIABETIC BLOOD.*

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It has been known for a long time that glucose disappears from blood which is kept under aseptic conditions after its removal from the body. This phenomenon, which is termed glycolysis, has been of considerable interest to investigators in view of its possible bearing on intermediary carbohydrate metabolism. During the last year attention has again been directed to this subject and an added interest given it by Denis and Giles (1) and also by Thalhimer and Perry (2). Both conclude, from data which they present, that the rate of glycolysis in diabetic blood is less than in normal blood and that the diminution of glycolysis is roughly in proportion to the degree of diabetic involvement of the individual studied.

Since the appearance of these suggestive papers, we have had occasion to measure the rate of glycolysis in the venous blood of non-diabetic individuals (arthritics). It was thought that a study of blood glycolysis in cases of chronic arthritis might contribute towards an understanding of the lowered sugar tolerance which has been found frequently to accompany this disease (3). On extending our observations to diabetic blood, we were unable, much to our surprise, to obtain evidence that such bloods suffered a loss of glycolytic power. In view of the importance of this

*The work here reported is part of a series of studies on chronic arthritis by Ralph Pemberton, M.D., of Philadelphia, in collaboration with Robert B. Osgood, M.D., of Boston. The expenses of the investigation were defrayed by contributions from various sources, including a number of patients.

subject in its suggested relation to the diabetic problem, we venture to present our results at this time.¹

LITERATURE.

Macleod (4) has reviewed the subject of glycolysis and from the extensive literature on the subject only those facts that seem pertinent to the problem of comparing the glycolytic power of different bloods will be considered here.

The disappearance of glucose from shed blood is generally ascribed to a glycolytic enzyme. This enzyme is seemingly restricted to the formed elements of the blood as no glycolysis occurs in serum or plasma (Macleod (5), Milne and Peters (6), Aibara (7)). Both erythrocytes (8) and leucocytes (9) contain the glycolytic enzyme, but it is reported as being absent from platelets (7). Glycolysis does not occur in laked blood (5, 7, 8). Macleod (5) found that glycolysis is decreased when potassium oxalate is present in concentrations sufficient to prevent coagulation of blood. Glycolysis is as rapid, however, in bloods in which coagulation has been prevented by sodium citrate (7) or hirudin (5) as in defibrinated blood.

Levene and Meyer (9) found that lactic acid is formed from glucose when leucocytes are suspended in buffered glucose solutions. Kraske (10) and also Kondo (11) report the same reaction during glycolysis in whole blood. Anrep and Cannan (12) have recently reported that an increased pH of the blood increases the production of lactic acid from glucose *in vivo* and to some extent *in vitro*.

In addition to the authors whose work was cited in the introduction Lépine (13) maintained that there is a diminished glycolytic power in diabetic blood. Eadie, Macleod, and Noble (14), however, failed to observe changes in the rate of glycolysis in bloods to which insulin had been added. Furthermore, blood drawn from animals after they had received injections of insulin exhibited no change in glycolytic rate.

Glycolysis appears then to be the reaction, glucose \rightarrow lactic acid, catalyzed by an enzyme present within the corpuscular elements of the blood. A comparison of the glycolytic power of different bloods involves a measurement of the velocity of this reaction and necessitates consideration of the factors which may influence the speed of the reaction.

¹ Himwich, Loebel, and Barr recently report that Tolstoi, working in the same laboratory, has been unable to find a delayed glycolysis in diabetic blood (Himwich, H. E., Loebel, R. O., and Barr, D. P., *J. Biol. Chem.*, 1924, lxx, 267. See also Tolstoi, E., *J. Biol. Chem.*, 1924, lxx, 69.)

Methods.

Each sample of blood was drawn under sterile conditions from the arm vein of the subject into a 50 cc. Erlenmeyer flask. The flask was stoppered with a sterile cotton plug and immediately taken to the laboratory and a sample removed for sugar determination. A piece of tin-foil was tightly fitted over the cotton plug and top of the flask containing the blood. The flask was then placed in an incubator maintained at 38°C.

During this preliminary manipulation the bloods were exposed to the air and lost CO₂. Consequently, the reaction of the bloods at the beginning of incubation was somewhat more alkaline than normal blood. In four bloods measured, the plasma pH before incubation was 7.92, 7.87, 7.88, and 7.78.

At intervals of usually 4, 6, and 24 hours, samples of the blood were removed from the flasks and the sugar content was determined.

At the end of the experiment the blood was transferred to beef broth. After 48 hours incubation, agar strokes were made from the broth. If no growth occurred on agar the blood was regarded as sterile.² It should be pointed out that the condition of the blood at the end of 48 hours incubation in broth may not represent its state during the early hours after it has been drawn. The presence of bacteria at this time does not necessarily mean that they were present at the beginning of the experiment, or if present, that they were in sufficient numbers to influence measurably the blood sugar. Despite utmost care in handling the bloods, it was difficult to avoid contamination from bacteria. However, comparison during the early hours of glycolysis, of the curves of sugar losses of the bloods showing final bacterial growth with those that were sterile gives no indications of differences ascribable to bacteria.

In some of the experiments the blood was defibrinated, in others coagulation was prevented by addition of potassium oxalate, sodium citrate, or heparin.

² We are indebted to Dr. John Eiman in whose laboratory and under whose direction this bacteriological work was done and to Miss A. M. H. Falck, who carried out all the tests for the presence of bacteria.

Blood sugar was determined by the Folin-Wu method (15). The color developed in this method when the blood sugar is very low is so faint that, even with the use of weak standards, accurate

TABLE I.
Glycolysis in Non-Diabetic Blood.

Blood.	Diagnosis.	Anticoagulant.	Blood sugar.					n
			0 hrs.	4 hrs.	6 hrs.	24 hrs.	Loss per hr. (first 6 hrs.).	
			mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
1	Arthritis.	Citrate.	83	13	13		11.7	0.308
2	"	"	90	32	30	†	10.0	0.183
3	"	"	91	47	19		12.0	0.260
4	Normal.	Defibrinated.	93	47	25	*	11.3	0.219
5	Arthritis.	Citrate.	99	42	24	*	12.5	0.236
6	Normal.	Heparin.	105	39	37	25	11.3	0.174
7	Arthritis.	Citrate.	153†	80	48	20†	17.5	0.193
8	"	"	155†	70	45		18.3	0.206
9	"	"	184†	138	76		18.0	0.147
10	Senile cataract.	"	209†	150	122	†	14.5	0.090
11	Malignancy.	Oxalate.	92	29	21		11.8	0.246
12	Normal.	"	93		25		11.3	0.219
13	"	"	94		45		8.2	0.123
14	Arthritis.	"	102	77	65	30*	6.2	0.075
15	"	"	102	26	26	24*	12.7	0.228
16	"	"	104		44	25†	10.0	0.143
17	Normal.	"	105	77	64	28	6.8	0.082
18	"	"	105	83	72	21	5.5	0.063
19	Arthritis.	"	184†	177	89	50	15.8	0.121
20	"	"	207†	133	97		18.2	0.126

*Sterile.

†Bacterial growth.

‡After glucose ingestion.

colorimetric readings are difficult. Results below 30 mg. per 100 cc. are to be regarded as approximations only.

RESULTS.

In Tables I and II are tabulated the results of glycolysis experiments on non-diabetic and diabetic bloods. The non-diabetic

bloods, with two exceptions, were obtained from normal individuals or from patients with chronic arthritis. In these tables are given the measurements of the initial glucose content of the bloods, the content after definite time intervals, and the average hourly

TABLE II.
Glycolysis in Diabetic Blood.

Blood.	Anticoagulant.	Blood sugar.					k
		0 hrs.	4 hrs.	6 hrs.	24 hrs.	Loss per hr. (first 6 hrs.).	
		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
1	Citrate.	143	67	38	†	17.5	0.221
2	"	144	66	39	20*	17.5	0.217
3	"	200	139	112	17†	14.7	0.096
4	Defibrinated.	208	127	110	*	16.3	0.106
5	Citrate.	242	108	73	*	28.2	0.200
6	"	270		153	20*	19.5	0.095
7	Heparin.	274	157	110	24	27.3	0.152
8	Citrate.	281	190	128	†	25.5	0.131
9	Defibrinated.	500	332	286	22*	35.7	0.093
10	"	529	361	335	*	32.3	0.076
11	Oxalate.	143		132	102	1.8	0.013
12	"	200	174	134	69*	11.0	0.067
13	"	200	165	127	13†	12.2	0.076
14	"	228	220	214	166	2.3	0.011
15	"	228	217	210	153	3.0	0.014
16	"	242		147	34	15.8	0.083
17	"	270		270	270	0.0	0.000
18	"	272	244	233	133	6.5	0.026
19	"	291	269	267	244	4.0	0.014

*Sterile.

†Bacterial growth.

rate of glycolysis during the first 6 hours. In a number of cases where the sugar concentration was not determined at exactly the 6th hour a curve representing sugar loss was constructed. The sugar level at the 6th hour was obtained by interpolation on the curve and this value used in calculating the hourly rate of glycolysis. On the assumption that the reaction, glucose \rightarrow lactic acid,

is monomolecular, the velocity constant has been calculated from the equation for a first order reaction:

$$K = \frac{1}{t} \log_e \frac{a}{a-x}$$

In this calculation a and x have been expressed as milligrams of glucose per 100 cc. of blood and t has been expressed in hours.

TABLE III.

Effect of Potassium Oxalate on Glycolysis in Diabetic and Non-Diabetic Bloods.

Blood.	Diagnosis.	Anti-coagulant.	Blood sugar.				
			0 hrs.	4 hrs.	6 hrs.	24 hrs.	Loss per hr. (first 6 hrs.).
			<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	Diabetes.	Heparin.	274	157	110	24	27.3
		Oxalate.	272	244		133	6.5
2	Arthritis.	Citrate.	184†	138	76		18.0
		Oxalate.	184†	177	89	50	15.8
3	Normal.	Heparin.	105	39	37	25	11.3
		Oxalate.	105	77	64	28	6.8
4	Diabetes.	Citrate.	270		153	20*	19.5
		Oxalate.	270		270	270	0.0
5*	"	Citrate.	200	139	112	17†	14.7
		Oxalate.	200	174	134	69*	11.0
6	"	Citrate.	143	67	38	†	17.5
		Oxalate.	143		132	102	1.8
7	"	Citrate.	242	108	73	*	28.2
		Oxalate.	242		147	34	15.8

*Sterile.

†Bacterial growth.

‡After glucose ingestion.

Bloods which were sterile at the end of the experiment are designated by an asterisk (*).

Contrasting results for a number of bloods are given in Table III. Two samples of the same blood were used: to one of these

potassium oxalate had been added and to the other sodium citrate. Examination of the results in this table, and in Tables I and II, reveals a decided difference in the effect of oxalates between diabetic and non-diabetic blood.

The rate of glycolysis in the blood of the same individual at different levels of initial sugar concentration has been studied by comparison of bloods drawn during a glucose tolerance test. In Table IV are given the results of three such experiments on arthritics.

TABLE IV.

Glycolysis in Non-Diabetic Blood Drawn during a Sugar Tolerance Test.

Blood.	Condition.	Anti-coagulant.	Blood sugar.			
			0 hrs.	4 hrs.	6 hrs.	Loss per hr. (first 6 hrs.).
			<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	Fasting.	Citrate.	99	42	24*	12.5
	After glucose.	"	155	70	45	18.3
2	Fasting.	"	90	32	30*	10.0
	After glucose.	"	153	80	48†	17.5
3	Fasting.	"	91	47	19	12.0
	After glucose.	"	83	13	13	11.7

*Sterile after 24 hours.

†Bacterial growth.

The experiment of adding glucose to blood was made. Its purpose was to study the effect of such additions on the rate of glycolysis.

Blood drawn from a normal individual was defibrinated by gentle stirring with a sterile glass rod. It was divided into three samples. To two of the samples glucose was added, in one a quantity sufficient to bring the amount in the blood to 264 mg. per 100 cc., in the other to 482 mg. per 100 cc. The three samples were then incubated and the glycolysis followed. At the end of 24 hours, the sugar in the unfortified blood had almost disappeared. Glucose was now added to this sample bringing the sugar

content of the blood to 151 mg. and the incubation continued for 24 hours without further loss of sugar. The three bloods were sterile at the end of the experiment. The results are given in Table V.

DISCUSSION OF RESULTS.

Variations of Initial Sugar Concentration.—Leaving out of consideration the bloods to which potassium oxalate has been added, examination of the tables will show that in both diabetic and non-diabetic blood the rate of glycolysis tends to increase with increases in the initial concentration of glucose. The increases in velocity

TABLE V.
Glycolysis in Normal Blood as Affected by Additions of Glucose.

Blood.	Blood sugar.				
	0 hrs.	4 hrs.	6 hrs.	24 hrs.	Loss per hr (first 6 hrs.).
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
1(a)	93	47	25	*	11.3
1(a) (after 24 hrs.).	151		145	135*	1.0
1(b)	264	207	186	35*	13.0
1(c)	482	425	360	215*	20.3

* Sterile.

are not, however, proportional to the changes in the substrate as would be necessary to satisfy the monomolecular velocity law. The falling off of k is not unusual in enzyme reactions. As Northrop (16) has recently shown in the case of trypsin, the equation of a first order reaction expresses the rate of change of substrate in enzyme actions only under very definite conditions, chosen to exclude side reactions and inactivation of the enzyme.

The effect on the velocity of the reaction of the amount of glucose present is shown by a comparison of the rates of glycolysis at different levels of blood sugar concentration. Glycolysis in bloods drawn at the height of hyperglycemia, following ingestion of 100 gm. of glucose, is appreciably faster than in the same bloods at a starvation level of blood sugar (Table IV). In the case where no rise of blood sugar followed the ingestion of glucose, the glycoly-

sis rate remained unchanged. The same thing was observed, though to a less extent, in the experiment where glucose was added to blood *in vitro*.

There is no suggestion whatever in these results that diabetic blood is lacking in glycolytic power. In fact, the rate of sugar loss is greater in diabetic blood of high sugar content than in normal blood. When diabetic and non-diabetic bloods of nearly the same initial sugar concentration are compared, the rate of glycolysis is approximately equal in the two groups.

Denis and Giles (1) following the general custom, have expressed their glycolysis results as *percentage* of sugar loss during definite time intervals. This is misleading when bloods of widely different initial sugar concentrations are being compared. Such expression is justified only if the rate of glycolysis is proportional to the glucose present; in other words, the velocity of the reaction must approximate that predicted by the monomolecular velocity equation. It has been shown that this condition is not met under the experimental procedure usually used to measure the rate of glycolysis. Denis and Giles do not state what means they employed to prevent blood coagulation and examination of their results suggests that oxalates were used. Oxalates may, as is shown above, have a decided effect on glycolysis in diabetic blood. In spite of this, many of their diabetic bloods, for example, Nos. 5, 16, 20, 26, 29 (Table II)³ show a greater rate of sugar loss during the first 6 hours than many of their non-diabetic bloods. This fact is masked when the results are expressed as *percentage* of sugar loss.

Variations in Enzyme Concentration.—In addition to variations in substrate, variations in the amount of enzyme present will cause velocity differences in enzyme action. Our data do not suggest that there is a lack of glycolytic enzyme in diabetic blood. In the first place, diabetic and non-diabetic bloods of the same sugar concentration show almost equal rates of glycolysis. In the second place, the glycolytic rate is much faster in diabetic blood than in normal blood brought to the same sugar concentration by the addition of glucose. The glycolysis rates of diabetic bloods Nos. 5, 6, 7, 9, and 10, Table II, are appreciably greater than the glycolysis rates of the corresponding normal bloods fortified by glucose (Table V).

³Denis and Giles (1), p. 741.

Enzyme Inactivation.—In common with other enzyme actions, glycolysis shows a decreased velocity with successive time intervals. With the data at hand it would be unprofitable to speculate on the causes of this flattening of the glycolysis velocity curve, further than to say that it does not seem to be entirely due to exhaustion of the substrate. In blood sample No. 1 (a) (Table V), the addition of glucose to the blood after 24 hours incubation was not followed by a resumption of glycolysis at the initial rate. In this case inactivation of the enzyme was complete and no further glycolysis took place even though glucose was added. The cause of the unusual effect of oxalates on the glycolytic enzyme and the seemingly greater effect of them on diabetic blood remains obscure.

Little comment can be offered on the results of Thalheimer and Perry (2) whose conclusions are so radically different from ours. Their data do not include the velocity of the sugar loss during the early hours of glycolysis. Until more is known of the conditions developing in blood during long periods of incubation, the influence of those conditions on the activity of the glycolytic enzyme remains uncertain. It would seem desirable in such experiments, to study glycolysis during the early hours, at a time when inactivation of the enzyme is presumably at a minimum.

SUMMARY.

1. The rate of glycolysis in bloods of widely different sugar content, incubated at 38°C. under sterile conditions, has been determined.
2. It is shown that the rate of glycolysis varies directly with the amount of sugar present but that there is a marked deviation from the rate predicted by the monomolecular reaction velocity equation.
3. No evidence was obtained indicating that there is in diabetic blood a diminution of glycolytic power. The effect of potassium oxalate in decreasing the rate of glycolysis has been found to be especially pronounced in diabetic blood.

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